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From the E.N T Department, University of Bergen, Norway
(Head Professor Emil Steen, M D)

A New Antitussive Agent of the Thiaxanthene Group Effect on Experimentally Induced Cough in Man

By

B. Ekholm and E. Steen

(Received A gust 30, 1965)

MOLLER NIELSEN *et al* (1962) and HOUGS *et al* (1965) have produced a substance, meprotixol (PINN) with a marked inhibitory effect on experimentally induced cough in cats and with little central depressant effect. Its formula and the results of animal experiments is described by HOUGS *et al* (1965) The antitussive effect of meprotixol in man has been reported by WINTHER (1966) and the present paper describes experiments to determine its antitussive effect in human subjects as compared with codeine on experimentally induced cough. Its effect on respiration was also determined.

Preliminary investigations showed that 90 mg/day of meprotixol for 21–38 days in 7 patients had no apparent toxic effects and 30 mg/day of meprotixol given to 10 healthy subjects in a double blind trial gave no symptoms which could be attributed the treatment

Technique

Antitussive effect

The technique described by BICKERMAN *et al.* (1954) and GRAVENSTEIN *et al.* (1960) was modified as follows (fig. 1) A 20 % solution of citric acid in water was nebulized by means of membrane compressor Pari Optimal, model Sternberg The apparatus was used without the inhalation mask, the discharge tube being placed vertically with its opening just in front of and on the level f the subjects lower lip. The subjects inhaled the aerosol f r one minute breathing quietly After each respiration, most of them had several attacks of coughing for the first ½ minute nd thereafter less often.

The cough response was recorded by means of a detached microphone (Bruel & Kjær condensor microphone No. 4131) placed at distance of ½ meter from the subject. A frequency analysis (Bruel & Kjær frequency analyser 2112, recorder No. 2305) of the sound

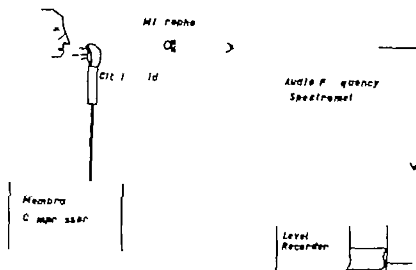


Fig. 1 Schematic representation f experimental procedure for inducing cough by inhalation of citric acid aerosol and for recording the cough by sound pressure measurement.

of the coughs showed that the peak pressure of the co ghs was about 90 db nd th back ground noise about 75 db. I order to obtain a straight baseline with the coughs marked as spikes from this line, the apparatus was adjusted to a zeroline corresponding to 80 db of sound pressure.

Effect on respiration

A method similar to that described by LUND & ERIKSSON (1959) was used. The respiratory minute volume and oxygen ptak per minute were recorded by means of the Godart pulmotest apparatus during 6 periods of 5 minutes each with 3-minute intervals.

Meprotixol was administered intravenously The subjects were given a constant infusion of physiological saline by intravenous drip. At the beginning of the third recording period, meprotixol was injected into the tube, unobserved by th subject.

Results

Reproducibility of cough response

Initial experiments on the reproducibility of the cough response were performed on 9 subjects. Each subject was given 3 successive exposures to citric acid inhalation at 10 minutes intervals. Figure 2 shows an example of the recording of cough responses in one subject. Table 1 lists the number of cough responses in 3 successive 1 minute periods of exposure at 10 minutes intervals, for each of the 9 subjects. It can be seen that although the responses varied considerably between subjects, the number of

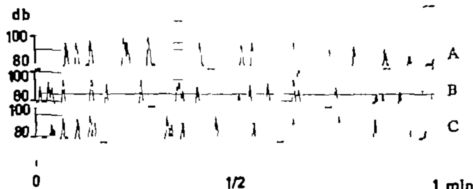


Fig. 2. Recording of cough response by sound-pressure measurement. The cough was induced by inhalation of citric acid aerosol for one minute and the response during one inhalation period was recorded as shown in A. The experiment was repeated twice with intervals of 10 minutes (BC). Time is plotted along the abscissa and the sound pressure in db along the ordinate. 80 db were used as zero line in order to obtain fairly straight baseline with sharply traced spikes for each cough.

responses per period was remarkably constant for each subject, Nr 6 being the only exception

When 4 subjects were tested daily for 6 days, a considerable day to day variation was observed (table 2). For this reason, in the determinations of an antitussive effect, a control trial was performed on each subject immediately before the administration of the testsubstance. Because

Table 1

Frequency of coughs during one-minute inhalation f 20 / citric acid aerosol. The figures indicate the number of coughs during the inhalation period Interval 10-minut between each trial

Subject No.	1st trial	2nd trial	3rd trial
1	17	17	1
2	23	21	25
3	10	10	9
4	19	19	17
5	22	21	22
6	39	21	22
7	23	20	22
8	14	15	13
9	7	7	7

Table 2

Day-to-day variation in frequency of coughs induced by inhalation f 20% citric acid aerosol. The figures indicate the umber of co ghs during inhalatio for 1 minute.

Subject No	1st day	2nd day	3rd day	4th day	5th day	6th day
1	15	8[1]	14	12	15	11
2	23	19	19	9[1]	18	17
3	37	22	19	11	18	17
4	19	17	17	17		

[1]) Experiment done immediately fter a meal.

the response on any one day tended to be constant as shown in table 1 it was necessary to perform only one control trial each day

Antitussive effect of intravenous meprotixol

After having determined the cough response to one control exposure to citric acid inhalation in 9 subjects, 10 mg meprotixol was administered intravenously The citric acid inhalation trials were then repeated 20 and 30 minutes later and again at 20–30 minute intervals for 1½–3½ hours.

Table 3

Antitussive effect of meprotixol (citric cid aerosol) Dose 10 mg intravenously

Subject No.	Antitussive effect[1])	Maximum effect fter
1	+++	20 min.
2	+++	30 min.
3	+++	30 min.
4	+?	40 min.
5	+++	30 min.
6	–	
7	–	
8	+	30 min.
9	+++	30 min.

[1]) – no antitussive effect
\+ slight antitussive effect (The cough response is reduced to approximately ⅔ f the daily basic response)
++ moderate antitussive effect (The cough response is reduced to approximately ⅓ of the daily basic response)
+++ marked antitussi effect (The cough response is reduced to approximately zero)

Table 4

Antitussive effect of meprotixol and codeine phosphate on cough (citric acid aerosol). Oral administration

Subject No.	Drug	Dose in mg	Effect
1	Meprotixol	10	++
	Codeine	25	+
	Meprotixol	10	+
	Codeine	25	–
3	Meprotixol	20	++
	Codeine	50	–
4	Meprotixol	20	–
	Codeine	50	+
5	Meprotixol	30	+++
	Codeine	75	++
6	Meprotixol	30	++
	Codeine	75	–
7	Meprotixol	20	++
	Codeine	50	++
8	Meprotixol	20	++
	Codeine	50	++
9	Meprotixol	20	++
	Codeine	50	++
10	Meprotixol	20	++
	Codeine	50	–
11	Meprotixol	30	+++
	Codeine	75	++
12	Meprotixol	10	–
	Codeine	25	++

The results are shown in table 3 It appears that 6 of the 9 subjects showed considerable reduction or complete suppression of the cough response with a peak effect 20 to 40 minutes after the injection

Antitussive effect of oral meprotixol

In a double blind experiment, the antitussive effect of tablets of 10 mg meprotixol was compared with that of 25 mg codeine phosphate. Each subject was tested in a cross-over experiment on two consecutive days receiving 1 2 or 3 tablets of meprotixol, followed the next day by an equal number of codeine phosphate tablets, or vice versa. It appears from

Table 5

Comparison of antitussive effect of meprotixol and of codeine phosphate on cough (citric acid aerosol). Oral medication. Various doses of meprotixol (5–20 mg) compared with a standard dose of 25 mg codeine phosphate.

Subject No	Drug	Dose in mg	Effect
4	Meprotixol	5	++
	Codeine	25	+++
7	Meprotixol	5	+++
	Codeine	25	+++
9	Meprotixol	5	+?
	Codeine	25	++
2	Meprotixol	10	+++
	Codeine	25	–
6	Meprotixol	10	++
	Codeine	25	–
12	Meprotixol	10	+++
	Codeine	25	+++
1	Meprotixol	15	+++
	Codeine	25	+++
8	Meprotixol	15	++
	Codeine	25	+++
11	Meprotixol	15	+++
	Codeine	25	+++
3	Meprotixol	20	+++
	Codeine	25	+++
5	Meprotixol	20	+++
	Codeine	25	++
10	Meprotixol	20	++
	Codeine	25	–

table 4 that 10–30 mg meprotixol showed an antitussive effect comparable to that of 25–75 mg codeine phosphate. The effect of meprotixol was manifest after 30 minutes and in 8 subjects an antitussive effect could still be observed 2 hours after administration.

When the subjects received 30 mg of meprotixol, they became drowsy This was even more marked with 75 mg codeine phosphate Thus these doses were considered too high for clinical use.

A new double blind test was then performed comparing 5 10 15 and 20 mg of meprotixol with a standard dose of 25 mg of codeine phosphate. The results are listed in table 5 Meprotixol in doses from 5–20 mg showed definite antitussive activity in 11/12 subjects, and in 10 of these 11

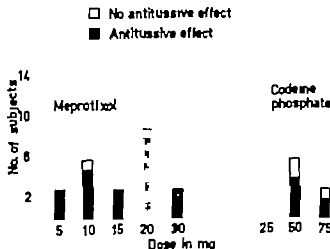


Fig. 3 Antitussive effect of meprotixol and codeine phosphate on cough induced by inhalation of citric acid aerosol. Oral administration.

subjects the effect continued during the 2 hour trial. 25 mg codeine phosphate was effective in 9 subjects.

Figure 3 illustrates the material from tables 4 and 5 The results are arranged by groups in columns according to the dose of meprotixol and codeine phosphate. It can be seen that meprotixol had an antitussive activity in 22 of the 24 subjects, while codeine phosphate had an antitussive effect in 17 out of 24 subjects. It is evident, moreover that meprotixol had an antitussive effect in a dose as low as 5 mg given orally It is also clear that failures occurred even with the highest doses, both with meprotixol and with codeine phosphate.

One of the subjects who received 30 mg meprotixol became markedly drowsy but was able to continue her work after the experiment. When she received 75 mg codeine phosphate, she became unable to continue her work after the experiment was concluded.

Effect on respiration

Eight subjects received 5 mg and two 10 mg meprotixol intravenously No effect was found either on the respiratory minute volume or on the oxygen uptake.

Discussion

The experiments indicate that meprotixol has a better and more reliable antitussive effect than codeine phosphate. Presumably 10 mg meprotixol 3 times a day is a satisfactory dose for clinical use.

The experiments also revealed that the irritant effect of inhaling an aerosol of citric acid was definitely increased when an antitussive drug was given presumably because this gave the citric acid an opportunity to penetrate deeper down into the airways. In those cases where the antitussive activity was most marked the subjects complained of a sore feeling behind and a little to the right of the sternum after the completion of the experiment.

With meprotixol, the subjects reported that inhalation of citric acid caused more marked irritation but this did not induce a cough response, although the subjects felt that they could cough if they wanted to do so. With codeine on the other hand, the irritation tended to subside. This might indicate a difference in the mechanism of action.

Summary

A new drug, meprotixol which in animal experiments has shown a marked antitussive effect, was investigated in man in order to elucidate its antitussive activity and effect on the respiratory centre. The results appear to confirm the findings in animal experiments. The antitussive activity of meprotixol was compared with that of codeine phosphate on cough induced experimentally by inhalation of citric acid. Meprotixol showed an antitussive effect equal to that of codeine phosphate. Unlike most antitussive agents of the opium alkaloid group meprotixol does not have a depressant effect on the respiratory centre.

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(Professor Jens Schou M D.)

Studies on the Subcutaneous Absorption in Mice IV Absorption of Carbohydrates with Different Molecular Weights from Connective Tissue

By

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(Received October 9 1967)

As a link in a series of investigations on the significance of the connective tissue ground substance for the subcutaneous absorption, the present paper deals with the absorption rate of three carbohydrates with different molecular weights.

Method

In halothane (fluothane ®) anaesthetized male albino mice, two symmetrical areas were marked out on the depilated skin of the back (cf SECHER HANSEN, LANGGÅRD & SCHOU 1967 a). A standard volume of 80 µl of distilled water containing about 0.5 µc of ^{14}C-mannitol (m.w 182), ^{14}C-sucrose (m.w 342) (The Radiochemical Center Amersham, England) or ^{14}C-inulin (m w 5000–5500) (New England Nuclear Corp., Boston, U.S.A.) was injected subcutaneously on the right side. In parallel groups the same solutions containing 40 I.u. of hyaluronidase (penetrase Leo ®) per dose were injected. In other animals 80 µl of 0.9 % NaCl containing the same tracer substances was injected. Five or 15 minutes later the animals were killed by decapitation. The two pieces of skin were excised and weighed and the difference between their total contents of radioactivity was measured (Packard Tri-Carb Liquid Scintillation Spectrometer) and expressed in per cent of the dose injected (residual radioactivity per cent).

Assuming a constant relative rate of disappearance from the injection zone (a process of first order) the absorption rate constant $K = \frac{\log. x_1 - \log. x_2}{(t_2 - t_1)\,0.4343}$ (KETY 1948 & 1949) was calculated for the periods 0–5 and 5–15 minutes.

Results

Figure 1a shows the residual radioactivity per cent originating from ^{14}C-mannitol, ^{14}C-sucrose and ^{14}C-inulin, 5 and 15 minutes after the in-

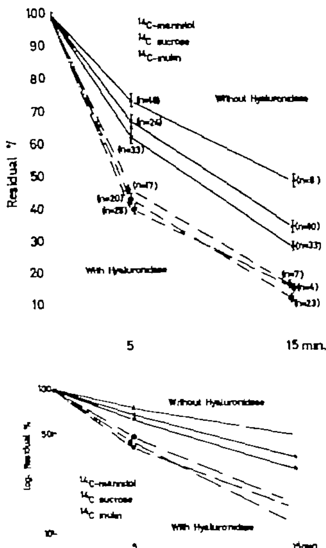


Fig. 1 Residual Radioactivity 5 or 15 minutes after subcutaneous injections of 80 µl of distilled water containing approximately 0.3 µc of 14C mannitol, 14C sucrose or 14C-inulin, without any hyaluronidase (upper curves) or containing 40 u. of hyaluronidase per dose (lower curves). The injections were given into the right of two symmetrical areas of depilated skin on the back of mice. The residual values are determined as the difference between the total radioactivity of the injected and uninjected side, and are expressed in per cent of the total dose injected. The standard error of the mean is indicated by vertical lines. n = number of animals.

The residual values are given in an arithmetic scale in upper part of the figure, while a semilogarithmic system is used in the lower part to demonstrate the same results.

Table 1

The Absorption Rate Constant of ^{14}C-mannitol, ^{14}C-sucrose or ^{14}C-inulin after subcutaneous injections of 80 µl of H_2O with or without 40 I.u. of hyaluronidase, or of 80 µl of 0.9% NaCl.

Tracer Substance	Absorption Rate Constant K				
	In H_2O		In H_2O + Hyaluronidase		In 0.9% NaCl
	0–5 min.	5–15 min.	0–5 min.	5–15 min	5–15 min.
^{14}C-mannitol	0.10	0.08	0.17	0.13	0.11
^{14}C-sucrose	0.08	0.07	0.18	0.10	0.11
^{14}C-inulin	0.06	0.04	0.16	0.11	0.10

jections. The rate of absorption for ^{14}C mannitol in water is significantly greater than for ^{14}C-sucrose in water which in its turn is significantly higher than for ^{14}C-inulin in water. When hyaluronidase is added to the solutions injected the three molecules disappear at the same rate from

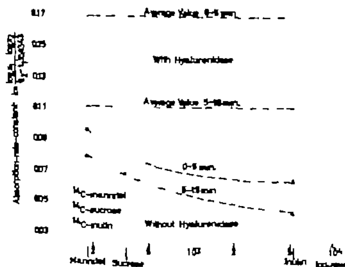


Fig. 2. The Absorption Rate Constant (first order process) of mannitol, sucrose or inulin is shown (ordinate) in relation to the logarithm of the respective molecular weights (abscissa). The absorption rate constant is calculated on the basis of the respective residual values 5 or 15 minutes after the subcutaneous injections of 80 µl of distilled water containing approximately 0.3 µc of the tracer substances, with upper curves or without (lower curves) the addition of 40 I.u. of hyaluronidase to the volume injected.

the injection site. In figure 1 b the same results are indicated in a semilogarithmetric system.

Table 1 shows the absorption rate constants calculated for the periods 0–5 minutes and 5–15 minutes for the tracer substances dissolved in water only and in water containing 40 I u of hyaluronidase per dose. In addition the rate constants are given for the period 5–15 minutes for the same substances dissolved in 0.9 / NaCl. It is seen that in the period 5–15 minutes, the rate constants for the three tracer substances in water after addition of hyaluronidase are not only identical but that they also correspond to the rate constants in a 0 9 / NaCl solution

In fig. 2 the molecular weights of the substances examined (abscissa log. molecular weight) are indicated in relation to the absorption rate constants (ordinate t [1]) for the periods 0–5 minutes and 5–15 minutes. In this way two parallel curves are obtained.

Discussion

It has been demonstrated that the connective tissue ground substance forms a barrier for the transport of colloid particles (DURAN REYNALS 1942 BALAZS 1961) and albumin (BLUMBERG & OGSTON 1956 LANGGÅRD 1964) as well as for crystalloids (SECHER HANSEN LANGGÅRD & SCHOU 1967 a og b) further more that this barrier effect depends on the concentration of hyaluronic acid (LAURENT & PETRUSZKIEWICZ 1961) and that it can be abolished by addition of hyalyronidase (SIMON & NARINS 1949 FORBES, DEISHER & PERLEY 1950 SECHER HANSEN, LANGGÅRD & SCHOU 1967 a & b). According to investigations *in vitro* (LAURENT & PETRUSZKIEWICZ 1961) the transport of different substances through the ground substance also depends on the nature of the molecules (i.e. their configuration, weight, charge etc.)

The present studies *in vivo* confirm that the diffusion delay which the ground substance exerts on different uncharged molecules with a uniform configuration increases with increasing molecular weight (fig. 1 and 2). It is worth noting, however that whereas the mannitol molecule is 30 times smaller than the inulin molecule, its absorption rate constant is only 1.5 times greater than that of inulin (table 1) It is also confirmed that the diffusion delay is due to the hyaluronic acid content, as after addition of hyaluronidase (fig. 1 & 2), table 1) the molecules disappear more rapidly and at the same rate It is further seen (table 1) that the absorption rate constants vary from the period 0–5 minutes to the period 5–15 minutes. The differen e is however the same for the different tracer substances.

The transport of molecules through the ground substance takes place

partly by diffusion (i.e. displacement of quantities of substance because of concentration differences) partly by filtration (i.e. displacement of quantities of liquids because of differences in pressure). The part of a substance which is removed by diffusion must depend on the molecular weight of the substance as well as on other molecular properties, whereas the part which is removed by filtration can be considered to be the same for all substances provided the injection volume and the solvent are kept constant.

When after addition of hyaluronidase the absorption rate constants are identical for mannitol, sucrose and inulin (fig. 1 table 1), this must signify that the removal of the substances injected from this greatly simplified system, in which the hyaluronic acid skeleton has been broken down, takes place by filtration. In the normal tissue, however, the diffusion delay of the connective tissue ground substance plays a considerable role (fig. 1 table 1). The distance between the curve for the absorption rate constants of the different tracer substances in a volume of water containing hyaluronidase and the corresponding curve for water only (fig. 2) is an expression of the diffusion barrier of the ground substance. As might be expected, this barrier appears to be greatest for the larger molecules. As far as the small molecules are concerned, the disappearance from the ground substance of the connective tissue mainly takes place by diffusion and in the case of the large molecules, mainly by filtration.

The fact that the absorption rate constants are the same in a 0.9 / NaCl solution as in aqueous solution after addition of hyaluronidase, indicates that the NaCl solution injected has in itself reduced the diffusion barrier. This finding which tallies well with previous observations (SECHER HANSEN, LANGGÅRD & SCHOU 1967b) implies presumably that in spite of the designation – *physiological saline* – should not be used as a solvent for investigations on the absorption from the subcutaneous tissue or from other connective tissue organs.

The curves in fig. 1b are not rectilinear. This indicates that within the experimental period, two or more processes participate in the absorption process. In this connection it is noteworthy that the course of the two curves in fig. 2 is approximately parallel. This indicates that the "extra disappearance" of the substances, taking place during the period 0–5 minutes is a constant for the different molecules and thus fulfills the criteria of a filtration process. During studies on absorption, a large number of investigators have demonstrated curves with an "initially downward convex" course. It seems justifiable at present to explain this phenomenon by an initially greater filtration probably due to mechanical conditions such as differences in pressure or perhaps injections directly into blood- or lymph vessels.

Summary

The rates of the subcutaneous absorption of ^{14}C mannitol (m.w 182) ^{14}C-sucrose (m.w 342) and ^{14}C inulin (m.w 5000–5500) were determined in mice. When the substances were dissolved in water the delay of absorption excerted by the connective tissue ground substance increased with increasing molecular weight. However after addition of hyaluronidase to the solutions, the rates of absorption were not only higher but identical for the different substances and further corresponded to the values obtained when 0 9 / NaCl was used as the solvent. Based on the findings it is suggested that after addition of hyaluronidase the absorption of molecules takes place solely by *filtration* whereas in the normal state absorption by *diffusion* plays a dominating role. Normal saline in itself seemed to reduce the barrier effect of the ground substance. The faster absorption observed shortly after the injections was apparently caused by a process fulfilling the criteria of a process of filtration

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Structural Characteristics of Oestrogen Binding in the Mouse Uterus Inhibition of 17β-Oestradiol Binding *in Vitro* by a Plant Oestrogen, Miroestrol

By

Lars Terenius

(Received September 11 1967)

A good deal of work has been devoted to structure-activity relationships of oestrogens. The biological activity studied has generally been growth stimulation of a target organ such as the uterus or vagina in rodents. The interpretation of this type of biological assay is obscured by the long time necessary between the time of administration of the oestrogens and the evaluation of the response. It can not therefore be decided whether the compound administered or its metabolites are the active principles at the target organ level. It has so far been found extremely difficult to demonstrate any response to oestrogens *in vitro*

The advent of radioactive oestrogens of high specific activity opened a new way to the problem It thus became possible to investigate the initial fate of physiological doses of systemically administered oestrogens. It was found that the synthetic oestrogen *meso*-hexoestrol (GLASCOCK & HOEKSTRA 1959) or the natural oestrogen 17β-oestradiol (JENSEN & JACOBSON 1962) are selectively taken up by the mammalian uterus and vagina. Subsequently it was found that 17β-oestradiol is taken up by the uterus of mice also *in vitro* (e g. STONE & BAGGETT 1965a). This uptake was found to be structure specific (STONE & BAGGETT 1965b TERENIUS 1966a). JENSEN *et al* (1966) found that an anti-oestrogen, U 11100A, inhibited the *in vitro* uptake of 17β-oestradiol in the rat uterus. TERENIUS (1966a) used oestrogens for analysis of inhibition Stereoisomeric pairs of oestradiol and hexoestrol with different oestrogenic potencies were found to block the uptake of labelled 17β-oestradiol in the mouse uterus differently The most oestrogenic isomer was the most effective uptake inhibitor This work was extended to highly potent synthetic oestrogenic

carboxylic acids, the structure of which radically differs from those of the natural oestrogens. It was found that these too cause inhibition (TERENIUS 1967a). Further, optical isomers of these acids and of 17β-oestradiol block the uptake of labelled 17β-oestradiol differently in the mouse uterus to an extent which increases with their oestrogenic activities (TERENIUS, unpublished). There is thus ample evidence that the uptake studied is closely connected with the physiological receptors.

All of the oestrogen left in the tissues after incubation does not represent specific uptake. For instance, the non-target tissue, the diaphragm, takes up considerable amounts of oestrogen. After incubation for 1 hour with radioactive 17β-oestradiol under the conditions used by the present investigator, the concentration ratios uterus/diaphragm are only around 4 (TERENIUS 1966a & 1967a). A great deal of the non-specific uptake of a radioactive oestrogen can be effectively washed out by a buffer which contains a large excess of the non-radioactive oestrogen. The specific uptake by the uterus, however, is largely retained during the wash-out. The outcome is that the tissue specificity increases since the concentration ratios uterus/diaphragm are between 25–50 (TERENIUS 1967b). In the present communication this uptake/washing procedure has been used.

Oestrogenic activity is found in a great many types of compounds, e.g. steroids, biphenolics and aromatic carboxylic acids. An oestrogen with a different and unique structure is miroestrol which can be isolated from a leguminous plant *Pueraria mirifica* native in Thailand (CAIN 1960). Its chemical structure has been established by X-ray crystallographic analysis and its physicochemical properties has been elaborately studied (TAYLOR, HODGKIN & ROLLETT 1960, BOUNDS & POPE 1960). Miroestrol was found to be a very potent oestrogen. These facts made it an attractive compound for the mapping out of structural requirements for oestrogen binding.

Materials and Methods

Radioactive 17β-oestradiol (oestra-1,3,5(10)-triene-3,17β-diol labelled with tritium at the 6 and 7 positions) was purchased from New England Nuclear Corporation. Its specific activity was 156 µc/µg. The radiochemical purity was about 98 % on chromatography on silica gel thin layers.

Non-radioactive 17β-oestradiol was obtained from Sigma Chemical Company. It melted at 173–4° and was homogenous in the thin layer chromatographic systems (table 1). Miroestrol was kindly donated by Dr. G. S. Pope at the National Institute for Research in Dairying, Shinfield, Reading, England. Since only a few mg of miroestrol was available, a stock solution in ethanol was prepared and the concentration of the solution calculated from the known molar extinction coefficients in UV light. The UV spectrum had all the characteristics described by BOUNDS & POPE (1960). The compound also moved as a single

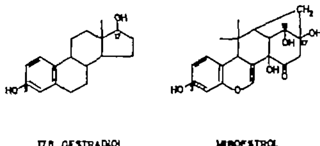


Fig. 1 Formula of 17β-oestradiol and miroestrol.

spot on all chromatograms (table 1). The chemical structures of miroestrol a d 17β-oestradiol are illustrated in fig. 1

Immature female mice of the N M.R.I strain were used. They weighed 8–10 g at the start of the uterotrophic assay or of the *in vitro* experiments. Uterotrophic activity was measured by the increase in uterine dry weight. The oestrogen was injected subcutaneously in 0.1 ml olive oil once da ly for 3 days (24 hours between each injectio). 24 hours after the last injection the animals were killed and the uteri dissected free. The uteri were dried at 100 for at least 4 hours and then weighed.

I vitro experiments were performed essentially as described previously (TERENIUS 1967 b). The uterus was divided at the cervix into two equal parts and 3–4 mg strips were cut out from the diaphragm. The incubatio medium was Krebs-Ri ger phosphate buffer pH 7 4 which contained 2% bovine serum lbumin. All incubations were carried out with air as the gas phase. The tissues were) incubated in 3 ml buffer for 1 hour at 37 ith 0 0005 μg radioactive 17β-oestradiol per ml (controls) the experimental flasks also contained the non-radioactive test oestrogen. The tissues were then b) transferred to other flask which contained 3 ml buffer and 0 05 μg non-radioactive 17β-oestradiol per ml and then incubated for 1 hour t 25 After incubatio the tissues were gently blotted and weighed wet. The tissues were individually sol bilised in hyamine ® (Packard Company) and the radioactivity measured by the liquid scintillation tech ique. The content of radioactivity is expressed on wet weight basis. The radioactivity taken up by the uterus under these i vitro conditions has been found to consist mainly of unchanged 17β-oestradiol (TERENIUS 1967b)

Results

The chromatographic mobility of miroestrol on silica gel thin layers in several chromatographic systems is shown in table 1 17β-oestradiol and oestriol were included as reference compounds. Miroestrol was found to move more like oestriol than like 17β-oestradiol

The log-dose/uterotrophic response curves of 17β-oestradiol and miroestrol are shown in fig. 2. The slopes are similar and 17β-oestradiol is about 5 times more uterotrophic than miroestrol the daily doses for ED50 s are 0.0055 μg and 0.025 μg respectively

The affinity of miroestrol for binding in the uterus was analysed indirectly by inhibition analysis *in vitro* For comparative purposes ana

Table 1

Thin layer chromatography of miroestrol, 17β-oestradiol and oestriol in various solvent systems. Thin layers were prepared from silica gel (Merck Kieselgel H). R_F values are given.

Compound	Solvent system[1]			
	I	II	III	IV
Miroestrol	0.07	0.45	0.37	0.06
17β-Oestradiol	0.52	0.65	0.59	0.41
Oestriol	0.14	0.41	0.28	0.10

[1]) I chloroform acetic acid (85 15) II ethyl acetate n-hexane:acetic acid:ethanol (72:13.5:10 4.5) (LISBOA 1966) III ethyl acetate cyclohexane ethanol (45 45 10) (LISBOA 1966) IV chloroform diethylamine (9:1).

logous experiments with non-radioactive 17β-oestradiol were carried out. Fig. 3 illustrates the effect of non-radioactive miroestrol and 17β-oestradiol on the binding of radioactive 17β-oestradiol in the uterus and the diaphragm. The two compounds give similar dose-response curves for the inhibition of the uterine retention Both compounds, in sufficient amounts, completely suppress the uterine retention down to the diaphragm level. The concentration giving 50 / inhibition of the uterine retention is 0 003 μg/ml for 17β-oestradiol and 0.1 μg/ml for miroestrol. 17β-oestradiol is thus about 30 times more active than miroestrol as inhibitor On a molar basis 17β-oestradiol is 45 times more active than miroestrol. Although not easily discernible in the figure, the very low retention in the diaphragm was not greatly affected by the test compounds.

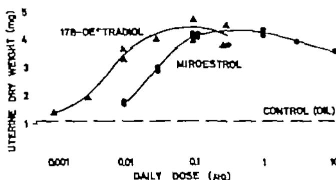


Fig. 2. Uterotrophic activities of 17β-oestradiol and miroestrol. Indicated doses were given daily on 3 successive days. Each point is the mean of at least 5 animals.

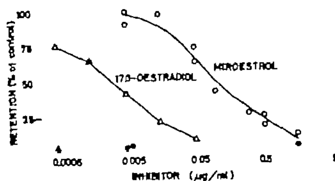


Fig. 3. Effect of various concentrations of 17β-oestradiol or miroestrol on the binding of radioactive 17β-oestradiol by the mouse uterus (open symbols). Closed symbols represent the retention by the diaphragm expressed in per cent of the control uterus. There were 4 half-uteri and 4 strips of diaphragm from 4 animals in each group.

Discussion

The uterotrophic potency of miroestrol in the present strain of mice was found to be about ⅓ of that of 17β-oestradiol (fig. 2). JONES & POPE (1960) injected the two compounds twice daily for three days into another strain of mice and found them to be roughly equiactive in producing increases in uterine wet weight. This discrepancy between our findings may depend on mouse strain differences or on differences in assay.

Fig. 3 shows that miroestrol has a comparatively low potency in inhibiting 17β-oestradiol uptake by the mouse uterus. The relative inhibitory activity of miroestrol in the *in vitro* test was 6 times less than expected from its oestrogenic activity. This discrepancy could be due to factors involved in the *in vitro* or in the *in vivo* assay. The *in vitro* system is by far the simplest, as it contains only the target tissue with the oestrogen in the surrounding medium. It seems unlikely that the low *in vitro* potency of miroestrol depends on degradation: on the contrary it appears to be comparatively stable in biological assays. For instance, miroestrol has been found to be 3 times more uterotrophic than the synthetic oestrogen stilboestrol when given orally in multiple doses, while the two compounds are equiactive after multiple subcutaneous injections. In the *in vivo* assays, however, many factors in addition to the interaction oestrogen-target tissue affect the response, e.g. the rate of absorption from the injection site, rate of degradation and excretion. It has indeed been found that, while being equiactive after multiple subcutaneous injections, after a single subcutaneous injection miroestrol is more uterotrophic than stilboestrol or 17β-oestradiol. The response elicited by miroestrol after the

single injection also lasts longer than that of the other two oestrogens (JONES & POPE 1960). In the present *in vivo* assay there was only one injection per day and a long-acting oestrogen such as miroestrol would have an advantage. Whether this explains the activity differences between the *in vitro* and *in vivo* assays remains to be seen

In unpublished experiments it has been found that the synthetic oestrogen *meso*-hexoestrol has ½ of the uterotrophic activity of 17β-oestradiol but has only $\frac{1}{15}$ of its uptake inhibitory activity in the present *in vitro* assay In the case of *meso*-hexoestrol it is also known that the compound *per se* is taken up by the uterus *in vivo* (TERENIUS 1966b) and *in vitro* (TERENIUS 1967b) However no comparable data on the duration of the effects of these two oestrogens are available.

As is apparent from fig. 1 the structure of miroestrol differs markedly from that of 17β-oestradiol. Miroestrol has 4 hydroxyl groups while 17β-oestradiol and stilboestrol only have 2 of these groups. As would be expected, miroestrol is quite polar and JONES & POPE (1960) actually found it to be at least 10 times more water soluble than 17β-oestradiol and even more water soluble than oestriol which has 3 hydroxyl groups. Miroestrol also had a lower chromatographic mobility than 17β-oestradiol in several systems (table 1)

The molecular dimensions, however of miroestrol and 17β-oestradiol are remarkably similar An X ray crystallographic analysis of miroestrol (TAYLOR, HODGKIN & ROLLETT 1960), revealed that both the distance between the 3-OH and 17-OH and that between the 3-OH and 18-OH are close to the distance deduced from molecular models between the 3-OH and 17-OH of 17β-oestradiol. The natural oestrogen oestriol can be derived from 17β-oestradiol by the substitution of a hydroxyl group at the 16 position. Like miroestrol, oestriol is a vicinal diol. The distance between the 3-OH and 16-OH of oestriol however differs considerably from the distance between the 3-OH and 17-OH In this connection it is of interest that oestriol gives a very shallow dose/uterotrophic response curve (HUGGINS & JENSEN 1955) while the dose response curve for miroestrol is normal. Miroestrol is also the more potent oestrogen.

Summary

Half-uteri and strips of the diaphragm from immature mice were incubated in a solution of 0 0005 μg tritium-labelled 17β-oestradiol per ml. After a subsequent washing the uterus contained 20 30 times more 17β-oestradiol than the diaphragm Isotope dilution with non radioactive 17β-oestradiol suppressed the selective uterine binding of radioactive

17β-oestradiol down to the diaphragm level A plant oestrogen miroe strol, similarly completely suppressed the uterine binding of 17β-oestradiol. This finding is discussed in terms of the oestrogenic potencies and chemical structures of 17β-oestradiol and miroestrol.

Acknowledgements

This work was made possible by the generous gift of miroestrol from Dr G S Pope. Mrs. Carola Engström gave competent technical assistance. The work was supported by the Swedish Cancer Society

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Determination of Sultiam (Ospolot ®) in Serum and Urine by Thin-Layer Chromatography Serum Levels and Urinary Output in Patients under Long Term Treatment

By

O. Vendelin Olesen

(Received July 31 1967)

Sultiamum INN (ospolot ®) is used in the treatment of epilepsy TANIMUKAI *et al* (1965) have demonstrated that there is a relation between the anticonvulsive effect of the drug and its inhibitory effect on carbonic anhydrase in the brain. The structural formula (see fig. 1) shows that chemically it can be grouped with the sulphonamides.

The aim of this investigation was to determine the concentrations of sultiam in the serum in patients under longterm treatment and to establish whether the drug has a tendency to accumulate in the blood. A 24-hour dosage of sultiam is usually divided into 2 doses, given in the morning and evening, and it is of interest to know the extent to which the serum concentrations vary during the interval between the two doses. Furthermore, we wished to examine how great a percentage of a given dose is excreted unchanged in the urine.

Sultiam is often used in combination with phenytoin and phenobarbital, and fig. 2b indicates that it would be difficult to separate sultiam from the two other antiepileptic drugs by partition between organic solvent and aqueous buffer We have therefore developed a thin-layer chromatographic method for the isolation and quantitative determination of the drug.

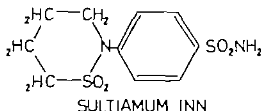


Fig. 1 2-(4-Sulphamoylphenyl)-tetrahydro-1,2-thiazinedioxide.

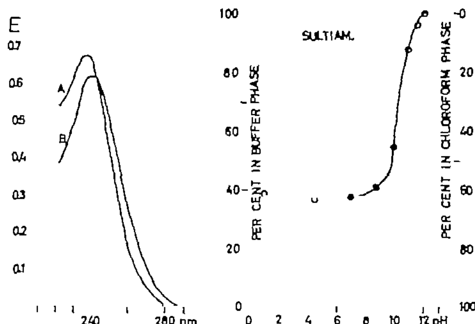


Fig. 2. Fig. 2 shows the extinction curve for sultiam (concentration approx. 20 mg/l) at pH > 10.5 (A) and pH < 9 (B) in the UV range.
Fig. 2b shows the distribution of sultiam between chloroform and aqueous buffer as a function of the pH of the buffer

Method

Serum

Sultiam was extracted from serum in the same way as described in the determination of phenytoin and phenobarbital (OLESEN 1967a & b).

3 ml of serum + 0.1 ml concentrated HCl are shaken vigorously for 10 seconds with 25 ml chloroform about 10 g of anhydrous sodium sulphate is dded, and shaking repeated for about 10 seconds. The chloroform extract is then filtered into burettes with glass stopcocks and attached funnel. 21 ml is transferred into 50 ml Erlenmeyer flasks, and evaporated to dryness in a water bath (60–70°) with stream of air. The residue is treated with 3 ml of methanol by shaking the flask in the water bath after cooling to room temperature the methanol extract is transferred to centrifuge tubes with a pointed bottom and the flask rinsed with 2 ml cold methanol, which is lso added to the tubes. The methanol is then evaporated and the inner surface of the centrifuge tubes rinsed with 2 ml chloroform, and again evaporated to dryness.

Two-dimensional thin-layer chromatography

Plates: 20 × 20 cm glass plates covered with 250 μm layer of Kieselgel G (Merck) to which Leuchtpigment ZS Super (Riedel de Haen) was added. The plates were activated at 110 for 15 minutes and stored t 50

Solvent I Methanol, glacial acetic acid, ether benzene, 1 9 30:60 /v

Solvent II Chloroform, methanol H_2O 83 17 1 v/v

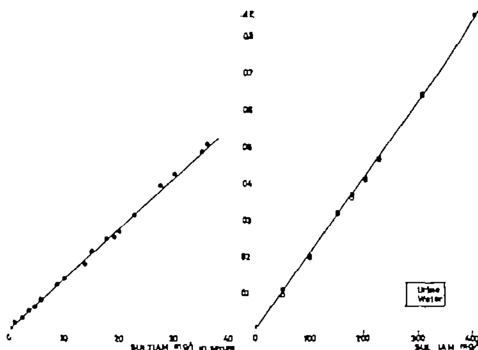


Fig. 3 Sultiam in serum and urine as a function of $\Delta E = E_{243}\text{-}E_{280}$, measured on methanol extract from thin-layer plates according to the methods described.

Chambers Both chambers were lined with filter paper and a vessel with concentrated ammonia water placed at the bottom of the chamber used for solvent II

Length of run 6 cm in each direction.

Reference solution 200 mg sultiam dissolved in 100 ml chloroform methanol 75:25 v/v

The residue in the centrifuge tubes was dissolved in 40 µl chloroform-methanol, 50 50 v/v and 10 µl applied to the plate. Four samples, or two duplicate samples, can be applied to a 20 × 20 cm plate, one in each corner 3 µl of the sultiam reference solution was applied at the mid-points of each side.

After development, the sultiam spots from the samples were localized by means of the reference spots under UV lamp (254 nm) and the area covered by spots marked off together with a blank of corresponding size, in which no fluorescent material was seen. The sultiam spots and blank spots were then scraped off and the powder poured into small centrifuge tubes 1 5 ml of methanol was added, the tubes closed with glass stopper and the powder extracted for about 2 minutes. One ml of the supernatant was transferred by means of a constriction pipette to semimicro quartz cuvettes (10 mm light path), and the extinction measured against the corresponding blank at 243 and 280 nm.

Calculation of sultiam concentration in serum

($E_{243}\text{-}E_{280}$ nm) × 72 mg/l sultiam in serum.

The conversion factor was found by adding known amounts of sultiam (1–40 mg/l) to various sera (see fig. 3) and carrying out duplicate determinations.

Urine

One ml of urine was shaken with 25 ml of chloroform for minutes in a centrifuge tube. After centrifugation, the aqueous layer was pipetted off and the chloroform filtered through a small amount of anhydrous sodium sulphate. Twenty-two ml of the chloroform extract was evaporated to dryness in an Erlenmeyer flask. The sultiam was transferred to centrifuge tubes by means of 3 ml hot and 2 ml cold methanol and the methanol extract evaporated to dryness. The residue was dissolved in 100 µl chloroform-methanol 1 1 /v and 10 µl was applied to the plate. Chromatography and spectrophotometric determination where performed as with serum.

Calculation of sultiam concentration in urine

$(E_{243}\text{-}E_{280}) \times 479$ mg/l sultiam in urine.

The conversion factor was found by adding known amounts of sultiam (50–400 mg/l) to respectively H_2O and urine from various subjects, and performing duplicate determinations (see fig. 3b).

Results

Recovery

The conversion factor for calculating the concentration of a pure methanolic solution of sultiam by means of $(E_{243}\text{-}E_{280})$ expressed in mg/l, was 27 7 which on correction for the following changes in volumes is 66 0 (3 ml serum was extracted, 21 out of 25 ml chloroform used ½ of the extract is applied on the plate. The spot is extracted with 1 5 ml methanol). The mean recovery from serum is thus 91 7 %, as the experimental found conversion factor was 72 (SD = 4 0 n = 18 duplicate determinations, see fig. 3a). A corresponding calculation for the urine analysis shows a recovery of 98.5 %. 27 7 × 25/22 × 10 × 1.5 = 472, the found experimentally conversion factor was 479 (SD = 14 4 n = 16 duplicate determinations, see fig. 3b).

Accuracy

The deviations between the double determinations on serum and on urine were calculated from the formula $S_A = \sqrt{\frac{\Sigma d^2}{2n}}$ where d is deviations between duplicate determinations and n is the number of duplicate determinations. For serum, S_A = 0.38 mg/l in the range of 0.7–12 mg/l, n = 36, and for urine, S_A = 2.9 mg/l in the range of 25–270 mg/l n = 29 The error did not seem to be dependable on the concentration within the examined range.

The Standard Deviation (SD) for single determinations on 20 different sera to with sultiam was added in an amount of 8 9 mg/l, was 0.44 mg/l.

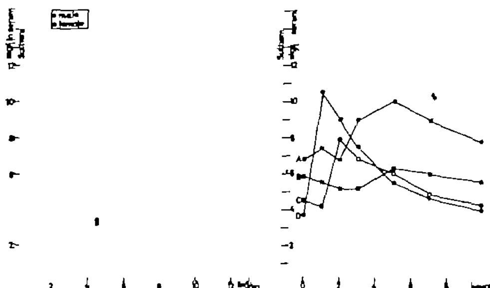


Fig. 4 Fig. 4a shows the relation between the 24-hour dose dministered in mg/kg and the minimum concentrations of sultiam in the serum in mg/l in patients under long-term treatment.

Fig. 4b shows the serum concentrations measured in the period between the administration of the morning and evening dose of the drug. Zero gives the time of the morning administration. A was treated with 400 0 400 mg sultiam daily B with 200 0 400 mg. C with 200 0 200 mg and D with 400 0 400 mg.

Specificity

When samples of serum and urine from patients or normal subjects not under treatment with sultiam were analyzed, no spots were found on the chromatogram in the region for sultiam. Rf × 100 for sultiam developed with solvent I was approximately 30 (phenobarbital and phenytoin about 50) Rf × 100 for sultiam solvent II was about 50 (phenobarbital 10, phenytoin 20) As shown in fig. 2a, the extinction curve in the UV range can be used for the identification of sultiam in the solution with a pH < 9 there is a maximum at 243 nm which shifts to 240 nm when the pH becomes higher than 10 5 while at the same time the extinction is increased by about 10 %. It appears from figs. 2a and 2b that there is a close relation between this phenomenon and the distribution of the drug between organic solvent and buffer A colour reaction for direct demonstration of sultiam on thin-layer plates was not found but if sultiam is irradiated with UV light on a thin layer plate, it is reduced and after this treatment a blue colour is produced when the material is sprayed with a solution of ferric chloride and potassium ferricyanide (ferric ferrocyanide).

Serum levels during long-term treatment

In 36 patients who had all been under sultiam treatment with 24-hour doses varying from 3 0 to 14 5 mg/kg for at least 6 months, blood samples were taken before the morning dose, i.e. approximately 14 hours after the last dose. The mean ratio mg/l/mg/kg was 0.56 (SD = 0.25). The serum content varied from 0 5 to 12.5 mg/l so that in no case was an accumulation of the drug found in the blood (see fig. 4a). In 4 patients A, B C, D (see fig. 4b), the variations in serum concentration were measured in the period between the morning and evening doses (10 hours) There was a considerable variation in the rates of absorption The peak of maximum serum concentration occurred 1–5 hours after the ingestion of a dose.

24-hour excretion of unchanged sultiam in urine

The 24-hour urine was collected in 27 patients and the concentrations of unchanged sultiam determined The 24-hour excretion expressed as a percentage of the dose administered, had a mean value of 32 %, SD = 13.3 % (maximum 69 %, minimum 17 %)

Discussion

DUHM *et al* (1963) in rat experiments with S^{35} labelled sultiam, showed that the absorption of the drug mainly occurred in the jejunum The rate of jejunal absorption is very variable, depending on a number of factors. The highly variable rates of absorption found in our four patients suggest that absorption also occurs in the jejunal in man Patient D (fig. 4b) with the steep absorption and elimination curve, had a 24-hour urine output of 3 1 l (a large fluid intake) while patient B with the flat absorption curve was very lethargic and a most constantly confined to bed.

In comparison with absorbable sulphonamides, both the maximum and minimum concentrations of sultiam in serum are strikingly low This can hardly be explained by a displacement in the distribution between the concentrations in tissue and blood to the advantage of the tissue, as the experiments by DUHM *et al* suggest that the opposite is the case.

Summary

A two-dimensional thin-layer chromatographic method has been described for the determination of sultiam in serum and urine.

The SD for single determinations on different sera was 0.44 mg/l,

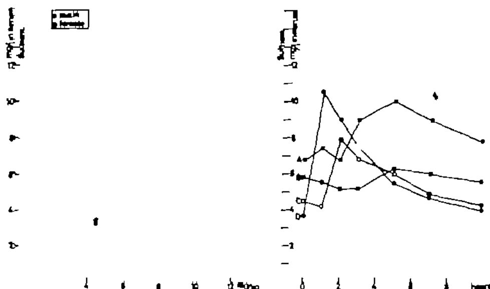


Fig. 4 Fig. 4a shows the relation between the 24-hour dose administered in mg/kg and the minimum concentrations of sultiam in the serum in mg/l in patients under long-term treatment.
Fig. 4b shows the serum concentrations measured in the period between the administration of the morning and evening dose of the drug. Zero gives the time of the morning administration. A was treated with 400-0-400 mg sultiam daily B with 200-0-400 mg, C with 200-0-200 mg and D with 400-0-400 mg.

Specificity

When samples of serum and urine from patients or normal subjects not under treatment with sultiam were analyzed, no spots were found on the chromatogram in the region for sultiam. Rf × 100 for sultiam developed with solvent I was approximately 30 (phenobarbital and phenytoin about 50). Rf × 100 for sultiam solvent II was about 50 (phenobarbital 10, phenytoin 20). As shown in fig. 2a, *the extinction curve in the UV range* can be used for the identification of sultiam in the solution with a pH < 9 there is a maximum at 243 nm which shifts to 240 nm when the pH becomes higher than 10.5 while at the same time the extinction is increased by about 10 %. It appears from figs. 2a and 2b that there is a close relation between this phenomenon and the distribution of the drug between organic solvent and buffer. A colour reaction for direct demonstration of sultiam on thin layer plates was not found, but if sultiam is irradiated with UV light on a thin-layer plate it is reduced and after this treatment a blue colour is produced when the material is sprayed with a solution of ferric chloride and potassium ferricyanide (ferric ferrocyanide).

Acta pharmacol. et toxicol 1968 26 29–34

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The Effect of Vasopressin on Myometrial Blood Flow in the Pregnant Rabbit

By

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(Received July 31 1967)

We have previously shown that the venous outflow from the rabbit uterus is markedly reduced following intravenous administration of vasopressin (CARTER, LEWIS & BENGTSSON 1966) We found a similar response in both pregnant and nonpregnant rabbits and were therefore anxious to obtain information about the effect of the drug on several components of the utero-placental circulation of pregnant animals. The present report concerns the effect of vasopressin on myometrial blood flow which we have determined by the ^{133}Xenon clearance technique. With this method it is possible to calculate absolute values for local muscle blood flow and it is in this respect superior to the study of ^{24}Sodium clearance (LASSEN LINDBJERG & MUNCK 1964) It has previously been applied to the study of myometrial blood flow in women (MUNCK, LYSGAARD PONTONNIER, LEFÈVRE & LASSEN 1964 LYSGAARD & LEFÈVRE 1965 LYSGAARD 1966 FALK, FORKMAN & LINDELL 1967).

Materials and Methods

Swedish white Lund rabbits with mean weight of 3 7 kg were used They were mated with fertile bucks and used 26 days later The mean length f gestation in this strain is about 31 days.

The animal was anaesthetized with pentobarbitone (mebumalnatrium 6 %, ca. 45 mg/kg, i.) and tracheotomy was performed. A polyethylene catheter (i.d. 1 14 mm) was tied into the right common carotid artery nd connected t pressure transducer (EMT 34 Elema-Schönander). The catheter and pressure chamber were filled with heparinized isotonic saline and the transducer was coupled t an electromanometer (EMT 31 Elema-Schönander) and an ink-jet recorder (Mingograph 81 Elema-Schönander), thus allowing

continuous registration of arterial pressure and heart rate throughout the experiment. Another catheter filled with heparinized saline was tied into the jugular vein on the same side.

Myometrial blood flow was determined by the following procedure. The abdomen was opened by a midline suprapubic incision to expose the uterus, and [133]Xenon (The Radiochemical Centre, Amersham), dissolved in isotonic saline to a concentration of approximately 10 mc/ml, was injected directly into the myometrium with a fine needle. Usually three injections of 0.03 ml were made in adjacent positions, in order to obtain a suitable initial counting rate of about 100 000 c.p.m. The isotope was always deposited in the antimesometrial wall opposite the placental site. The abdominal incision was closed with a clamp or covered with a moist swab

The disappearance rate of the isotope was recorded with a scintillation detector (IDL) with crystal diameter of 5 cm, fitted with a wide angle collimator and placed 5–10 cm above the abdomen. The detector was coupled to linear precision ratemeter (Picker Dual Ratemeter) and the output recorded on logarithmic potentiometer writing on linear paper (Beckman). Myometrial blood flow in ml/100 g tissue/min was calculated from the initial slopes of the disappearance curves, using the method and constants of LASSEN, LINDBJERG & MUNCK (1964). Two separate determinations were made in each animal one before and one during vasopressin administration.

Vasopressin

Synthetic lysine-vasopressin (postacton ®, Ferring) was administered as an infusion through the jugular catheter with the aid of an infusion pump. Previous experience indicated that rapid injections were undesirable as they lead to sudden and violent fluctuations in blood pressure (CARTER, LEWIS & BENGTSSON 1966). The drug was therefore given at a rate of 30 mIU/min. and the myometrial blood flow determined with new isotope injection after 10 minutes of infusion, with the pump still running.

Large doses of vasopressin administered to pregnant rabbits cause foetal death apparently associated with placental damage (KNAUS 1926 BENGTSSON 1957). In order to ascertain whether the foetuses survived the relatively lower doses used in the present experiments, we examined them immediately after registration of the second clearance curve was completed

Results

Individual values for myometrial blood flow (MyBF) are presented in table 1 The seven values obtained before vasopressin infusion give a mean MyBF of 24 ml/100 g/min This may be taken as representative of the resting flow under the conditions of the experiments. In all cases, the values obtained during vasopressin infusion were much lower giving a mean MyBF of 7 ml/100 g/min. The difference between the means is statistically significant ($p < 0.01$) The reduction in MyBF for a single experiment is illustrated in fig. 1 which shows the [133]Xenon disappearance curves obtained from the same rabbit before and during infusion of the drug.

Carotid arterial pressure and heart rate were monitored throughout each experiment in order to establish the general circulatory response to

Table 1

Values for myometrial blood flow (MyBF) in ml/100 g tissue/min. obtained with the [133]Xenon clearance technique in the resting state and after infusion of vasopressin (30 mIU/min.) for 10 minutes.

Rabbit No	MyBF without vasopressin	MyBF during vasopressin infusion
119	6	2
165	20	6
162	34	15
164	25	11
178	40	1
180	16	6
181	25	10
means	24	7

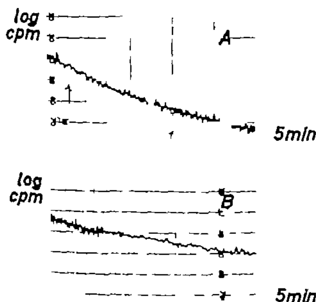


Fig. 1 [133]Xenon clearance curves from a pregnant rabbit. The isotope was injected intramyometrially and MyBF calculated directly from the slope of the curves, a steeper slope giving a higher value for MyBF. A. First isotope injection, before vasopressin, MyBF = 16 ml/100 g/min. B. New isotope injection, after ten minutes of vasopressin infusion, MyBF = 6 ml/100 g/min.

the vasopressin infusion and to manipulation of the uterus and abdominal wall, during injection of the isotope solution. The administration of vasopressin resulted in a pressor response and bradycardia. The mean increases in systolic and diastolic pressure were 23% and 25%, respectively and the mean reduction of heart rate 46%. Manipulation had no perceptible effect before vasopressin treatment. A slight fall in carotid arterial pressure was registered during most of the isotope injections made during infusion of vasopressin.

A total of thirty-nine foetuses were examined after vasopressin infusion. Of these, only seven failed to give a gasping reflex, either spontaneously or when mechanically stimulated. They were obtained in three rabbits in which we also found a certain amount of blood in the uterus. In all cases, however, at least half the litter appeared viable.

Discussion

The results presented above indicate that there is a marked decrease of myometrial blood flow in pregnant rabbits during intravenous infusion of vasopressin in the dose administered. This finding demonstrates that the myometrial component of the utero-placental vasculature contributes to the decrease in total uterine blood flow which we have previously shown after slightly higher doses of the drug (200 mIU/min. for 5 mins. intravenously; CARTER, LEWIS & BENGTSSON 1966). In a subsequent paper we shall show that the placental blood supply is also affected by vasopressin.

The reduction in myometrial blood flow during vasopressin infusion was accompanied by a rise in systemic arterial pressure and bradycardia. These responses to vasopressin are well known and have been described in the rabbit by several investigators (SHARPEY-SCHAFER & MACDONALD 1926, HOLTZ 1932, BROWN, MCLEAN & MAEGRAITH 1939, HARRIS 1948). The pressure rise is attributed to an increase in peripheral resistance resulting from vasoconstriction. The slowing of the heart is probably produced through the vagus, as a result of this rise, and also by the coronary constriction and myocardial depression resulting from the direct action of the drug (MCDOWALL 1938). Since these responses were well-marked in our experiments, it seems likely that there was a marked vasoconstriction and that the observed reduction in myometrial blood flow can be attributed to constriction within the uterine vascular bed.

A number of previous studies also suggest that the uterine vascular bed constricts in response to vasopressin. ROBSON & SCHILD (1938) made a

constant rate perfusion of the uterus *in situ* in pregnant and steroid treated, spayed cats, and registered an increase in perfusion pressure when vasopressin (0 05–1 0 IU) was injected into the uterine artery They also recorded a decrease in uterine volume by means of an oncometer WAISVISZ (1965), working with *in vitro* preparations of intact human uteri, reported an increase of perfusion pressure after administration of pitressin (0 1 IU)

It is unlikely that uterine contractions play a role in the action of vasopressin on myometrial blood flow Contrary to the human uterus, the rabbit uterus does not contract in response to vasopressin *in vivo* large doses usually cause a prolonged depression of uterine activity whilst smaller doses have no observable effect (WEINSTEIN & FRIEDMAN 1935 MORGAN 1937 ROBSON 1937 FUCHS 1964)

Large doses of vasopressin cause foetal death when administered to pregnant rabbits (KNAUS 1926 BENGTSSON 1957) In the present experiments, the majority of the foetuses remained viable after vasopressin infusion. It is not possible to say whether the death of the exceptions was due to the drug or merely to placental dislocation, resulting from manipulation of the uterus. Even if the dose used here was not lethal for the foetuses, it was nevertheless greater than that likely to be released from the pituitary under physiological conditions. It is not impossible that physiological levels of the hormone cause subtler changes in the utero-placental circulation and play a role in its regulation The investigation of this problem must, however await the development of techniques of measurement which are more sensitive than those currently available.

Summary

Myometrial blood flow was measured in anaesthetized pregnant rabbits by the [133]Xenon clearance method before and during infusion of vasopressin (30 mIU/min. for 10 mins. intravenously). The mean flow values before and during infusion were 24 and 7 ml/100 g tissue/min. respectively ($p < 0.01$). The significance of this result is discussed.

Acknowledgements.

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From the Department of Pharmacology and Toxicology The Royal Veterinary and Agricultural College, Copenhagen

Detection by Thin-Layer Chromatography of Organophosphorus Insecticides in Acutely Poisoned Rats and Chickens

By

J Beck and M. Sherman[1])

(Received May 26, 1967)

A screening method using thin layer chromatography for detecting six commonly used organophosphorus insecticides has been developed. This procedure is offered as a supplement to the determination of cholinesterase activity used as an indication of animal poisoning with organophosphorus insecticides (FRIEDBERG & SAKAI 1958 KARLOG & POULSEN 1963) Dichlorvos, dimethoate, malathion, methyl-parathion, parathion and trichlorfon were separated by means of solvent systems and chromogenic sprays according to the methods described by STANLEY (1964), SALAMÉ (1964) and EL REFAI & HOPKINS (1965) and adapted as discussed below

It is possible to separate and identify these insecticides from the contents of the alimentary canals of chickens and rats killed following oral administration.

Material and Methods

Thin-layer chromatography basic equipment from Desaga, Heidelberg, was used with the following solvent systems 1) Chloroform-acetone (1 + 1) 2) Chloroform-carbon tetrachloride (1 + 1). Acetonitrile was used for extraction

Reagents

Silica gel G for thin-layer chromatography (Merck).
Alcoholic sodium hydroxide 4 4 g sodium hydroxide dissolved in 5 ml distilled water and diluted to 100 ml with 96 % ethyl alcohol.
Silver nitrate-R 1 g silver nitrate dissolved in 25 ml distilled water and diluted with acetone to 100 ml. (Stored for 2 weeks before use).

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Table I

Doses in terms of actual ingredients of six organophosphorus insecticides administered orally to chickens and rats.

Insecticide	Dosage mg/kg	
	Chicken	Rat
Dichlorvos	50	150
Dimethoate	100	1000
Malathion	1000	2000
Methyl-parathion	40	40
Parathion.	20	20
Trichlorfon	100	1000

minutes before filtration. The filtrates without further cleaning were spotted in volumes of 100 µl or less and analyzed by thin-layer chromatography in the two solvent systems described above.

Results

The reference grade insecticides as separated on thin-layer by the two solvent systems are shown in fig. 1 Both chromogenic sprays were able to detect between 0.2 and 0.5 µg of the insecticides.

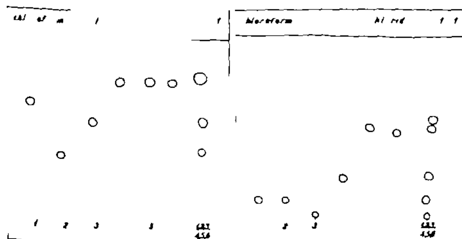


Fig. 1 Thin-layer chromatograms on silica gel in the solvent systems chloroform-acetone (1 + 1) and chloroform-carbon tetrachloride (1 + 1)
1 dichlorvos, 2. trichlorfon (detected with silver nitrate), 3. dimethoate, 4. malathion (detected with palladium chloride) 5. parathion, 6. methyl-parathion (detected with sodium hydroxide), all in an amount of 0.5 µg.

Table 2

Detectability of organophosphorus insecticides by thin-layer chromatography in animal digestive tracts.

Insecticide	Chicken: Crop	Chicken: Gizzard and intestine	Rat: Stomach	Rat: Intestine
Dichlorvos	+	+	+	+
Dimethoate	+	−	+	−
Malathion	+	−	+	−
Methyl-parathion	+	+	+	−
Parathion	+	+	+	+
Trichlorfon	−	+	+	−

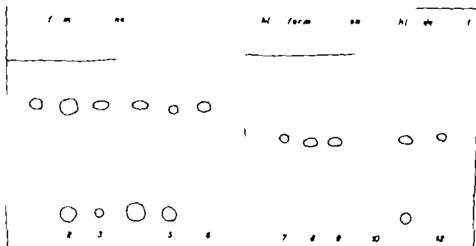


Fig. 2. Thin-layer chromatograms on silica gel of acetonitrile filtrates of samples from animals, poisoned with dichlorvos (2–5 solvent system chloroform + acetone, detected with silver nitrate) and methyl-parathion (8–11 solvent system chloroform + carbon tetrachloride, detected with sodium hydroxide).
1. 1 µg dichlorvos 94 %, 2. 50 µl chick crop filtrate, 3. 50 µl rat stomach filtrate, 4. 50 µl chick gizzard-intestine filtrate, 5. 50 µl rat intestine filtrate, 6. 4 µg dichlorvos 24 % emulsifiable concentrate, 7. 1 µg methyl-parathion 94 %, 8. 50 µl chick crop filtrate, 9. 50 µl rat stomach filtrate, 10. 50 µl rat intestine filtrate, 11. 50 µl chick gizzard-intestine filtrate and 12. 2 µg methyl-parathion 48 % emulsifiable concentrate.

When all six compounds are spotted together in the chloroform acetone system, dichlorvos is lost and parathion, methyl-parathion and malathion come out at the same spot. It appears that dichlorvos, when spotted together with parathion or methyl-parathion cannot be detected by spraying with silver nitrate.

All animals died between 5 minutes and 4 hours after oral administration of the insecticides. The organophosphorus insecticides were detected in the alimentary canals of all treated rats and chickens (table 2). In some rats, the compound was found in the stomach and not in the intestine and in others, they were found both in the intestine and the stomach. In some chickens the compound was found only in the crop or only in the gizzard and intestine

Figure 2 shows the detection of dichlorvos and methyl-parathion

Discussion

The proposed thin-layer chromatography procedure is a supplement to the verification of poisoning by anticholinesterase organophosphorus insecticides, where the cholinesterase activity determination before and after reactivation with specific reactivating oximes in blood and brain tissue are helpful but possibly not definite. In parathion poisonings for example, which are still the most common cholinesterase activity can be spontaneously reactivated in inadequately stored tissue (KARLOG & POULSEN 1963) and the causal agent overlooked.

In acute poisonings, the alimentary canal content is a suitable subject for analysis because of slight or no metabolism and the presence of a relatively high concentration of poison. Thus, this simple method can be used as a qualitative screening procedure Subsequently the liver tissue after cleaning can be analyzed quantitatively for the parent compound and its known metabolites.

Summary

A qualitative thin-layer chromatographic method is described for separating dichlorvos, dimethoate, malathion, methyl-parathion, parathion, and trichlorfon after a simple acetonitrile-extraction of samples.

The procedure has been effective for the alimentary canal contents of rats and chickens, poisoned with twice the LD50 doses of these insecticides.

Acknowledgements

We would like to thank A/S Agro-Kemi A/S Dansk Shell, A/S Nordisk Alkali Biokemi and American Cyanamid Co for the reference grade and commercial formulations of the organophosphorus insecticides. One of the authors (M.S.) is particularly indebted to the U.S Educational Foundation in Denmark for making his stay possible.

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Investigation on the Toxicity of Small Chronic Doses of Tannic Acid with Special Reference to Possible Carcinogenicity

By

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(Received June 7 1967)

In 1925 DAVIDSON suggested the use of tannic acid in the local treatment of burns, but not until World War II was it realized that absorption of tannic acid from large ulcerations of wounds might have a hepatotoxic effect (BAKER 1943 CAMERON *et al* 1943 ERB *et al.* 1943 FORBES & EVANS 1943 WELLS *et al.* 1942) The hepatotoxic effect was also demonstrated experimentally in animals.

In 1949 KORPÁSSY & KOVÁCS showed that parenteral administration of tannic acid in white rats induced cirrhosis of the liver showing a condition resembling Laennec's cirrhosis in man. Further investigations by KORPÁSSY & MOSONYI (1950) showed that tannic acid USP 1.5 / injected subcutaneously in doses of 150 to 200 mg/kg to rats every fifth day for 290 days not only induced cirrhosis of the liver but also hepatomas and cholangiomas.

Oral treatment for more than 180 days also resulted in cirrhosis with a dose which was several times greater than the usual parenteral dose (KOLTAY & KORPÁSSY 1951)

Later investigations by MOSONYI & KORPÁSSY (1953) and by KORPÁSSY (1959) showed that tannic acid enhanced the carcinogenic effect of 2 acetamidofluorene.

In 1960 KIRBY found that condensed tannins caused sarcomas at the site of injection and also liver tumours in rats and mice, while extracts of hydrolysable tannins caused only liver tumours. The rats were given subcutaneous injections corresponding to 0.5 mg tannin extract per week for 12 weeks and the mice subcutaneous injections corresponding to 0.125 mg extract per week for 12 weeks.

The repeated subcutaneous administrations of tannic acid resulted in necrosis of the skin at the site of injection. Although fairly large skin

ulcers were produced in animals treated for long periods, the ulcer healed rapidly and did not seem to influence the general health of the animals. In this series no tumours arose from the margin of ulcers or from the healed scars.

Daily painting with 5 % fresh tannic acid solution on the back after the skin had been burned to ulceration caused no remote effects, such as *cirrhosis, hepatomas,* or *cholangiomas.*

The doses of tannic acid used in the investigations mentioned above were all rather large, but the results suggest that prolonged use of tannic acid may have toxic effects.

At present, not a few pharmaceutical preparations contain tannic acid including preparations for intramuscular or subcutaneous injection. Generally only small doses are used, but their possible toxic effect remains unknown. The present investigation was made in order to determine the effect of tannic acid in the small doses used as recommended.

In the experiments reported here tannic acid was used as present in the *vitamin B_{12} preparation betolvex ®. This preparation is recommended* for the treatment of pernicious anaemia with intramuscular injections of 1 ml every second or third month.

In order to ensure that absorption of the preparation from the injection site had taken place, the concentration of vitamin B_{12} was determined in the spleen, kidney and liver of the killed animals.

Methods

Young C_3H/A mice weighing 19–22 g at the beginning of the experiment were used. The animals were kept in single cages and fed on mouse biscuits consisting of: Fish meal 9.0 %, wheat (ground) 76.5 %, linseed meal 3.0 %, lucerne meal 9.0 %, skim-milk powder 2.25 %, and medical train oil 0.25 %, mixed to a thick mash, then made into biscuits and dried for 6 hours at 100°.

Water ad libitum.

180 mice were used in the experiment. The mice were divided into three groups each containing 30 males and 30 females. Animals in group I were treated with saline and used as controls, those in group II with the suspending medium used for the betolvex preparation but without vitamin B_{12}, and those in group III received betolvex ®. Every 14th day the animals were injected intramuscularly with 0.01 ml of either saline, solvent, or betolvex ® alternatively in the right and left hind leg. 1 ml of betolvex ® contains 2.5 mg cyanocobalamin tannium suspended in 2 % aluminium monostearate gel in sesame oil. 2.5 mg of this *complex corresponds to 1 mg cyanocobalamin and 1.5 mg tannic acid.*

The tannic acid used was nutgall tannic acid, a complex of esters of D-glucose with gallo-tannic acid and galloyl gallo-tannic acid. It corresponds to tanninum (Ph. Nord.), acidum tannicum (Brit. 32) and to tannic acid (U.S.A. 47). The total dose of tannic acid was 1.5 mg/kg/month.

All surviving mice received 26 injections during 12 months. After a further observation period of 6 months, the animals were killed, and autopsy was performed including removal

of lungs, liver spleen, and kidneys. Furthermore, the sites of injection were removed for histological examination. Liver and spleen were weighed some of the material was fixed in formalin, imbedded in paraffin, sectioned, and 4 μ sections were stained with haematoxylin-eosin and Sirius Red. Some of the liver spleen and kidneys was homogenized and the vitamin B_{12} content determined as described in Ph. Nord. Vol. IV (1963) with lactobacillus leichmanii as a test object[1].

Results

There was no difficulty in injecting the substance intramuscularly In no case did local reactions or local tumours develop The experiment lasted 18 months, and each animal surviving that period received 26 injections. At each injection the animals were weighed and most of them gained 1–2 g in weight during the experiment. The weight curves for the three groups were parallel

During the experiment 6 animals of group I died (1 from mammary cancer 1 from lymphatic leukaemia, and 1 from a tumour in the scapular region), 6 animals of group II died (1 from mammary cancer 1 with a doubtful hepatoma, and 1 with a fibrosarcoma in the abdomen) and 8 animals of group III died (3 from mammary cancer 1 with a fibrosarcoma in the abdomen, and 1 probably from lymphatic leukaemia) Other causes of death were pneumonia and enteritis but some of the animals were in such a poor state that diagnosis was impossible.

There was no statistically significant difference in survival time between the three groups.

The average weight of the livers of the three groups was as follows Group I, 1 28 g (S = 0.27) group II 1 28 g (S = 0 39), and group III 1 48 g (S = 0.36) The average weight of the spleens was Group I, 0 09 g (S = 0.03), group II 0.08 g (S = 0 02), and group III 0 07 g (S = 0 03).

As tannic acid in toxic doses is primarily cirrhotogenic and consequently capable of inducing atrophy of the liver the weights of the liver indicate that the doses used in group II and III have hardly been cirrhotogenic.

Histological examination of the livers showed cirrhosis in one mouse only (group I the control group). In some of the mice there were signs of fatty degeneration of the liver and in some cases regional derangement of the histological structure. These changes were found with the same frequency in all three groups of animals.

In group II and III identical changes were seen at the sites of injection,

[1] The microbiological vitamin B_{12} determinations has been done by the Microbiological Laboratory of Dumex Ltd., Copenhagen.

Table 1

The average content of vitamin B_{12} in liver, spleen, and kidneys in mice.

Group \ Organs	n[1]	Vitamin B_{12} in µg/g organ		
		Liver	Spleen	Kidneys
I Saline	21	0.31 ± 0.06[2]	0.19 ± 0.04	0.43 ± 0.08
II Solvent	23	0.36 ± 0.13	0.19 ± 0.06	0.48 ± 0.18
III Betolvex ®	56	0.50 ± 0.15	0.33 ± 0.21	1.76 ± 0.90

[1]) n = No. of animals. [2]) Standard deviation.

viz. sequestration of the muscles and development of small or large sometimes multilocular lacunas often containing amorphous substance, probably lymphocyte accumulations were seen close to the sites of injection, but otherwise there were no signs of toxic effects. In no case did malignancy develop around the canals of injection.

The vitamin B_{12} content of the organs from some randomly picked animals was found as shown in table 1.

Discussion

The increase of vitamin B_{12} in the organs shows that a sufficient absorption from the site of injection has taken place.

Each mouse received 1.5 mg tannic acid/kg/month. The doses used in pharmaceutical preparation for parenteral use are considerably smaller, for example, the maintenance dose in treatment of pernicious anaemia in man is 1 ml betolvex ® every third month, i.e. a man weighing 70 kg receives about 0.02 mg betolvex ®/kg every third month. KORPÁSSY *et al.* gave 150–200 mg/kg to the animals, i.e. the quantity of tannic acid given to man in common therapy is considerably smaller than the dose used in the mouse experiments and much less than the doses used to demonstrate the carcinogenicity in rats.

It should be mentioned that the daily intake of tannic acid is generally rather high. 100–500 mg is found in a cup of tea, cocoa, or coffee. "Light" clarets contain 0.1–1.15 ‰, "heavy" ones 0.2–0.3 ‰.

The present experiments show that in the small doses in which tannic acid is administered there is no risk of liver damage or carcinogenesis. This is in contrast to the results in investigations in which much larger doses of tannic acid were used.

Summary

Mice were injected every second week intramuscularly with 0 75 mg tannic acid/kg for 18 months. Only a weak local reaction was seen. The injected mice did not differ from the corresponding control groups no signs of liver damage, particularly cancer were found.

Acknowledgement

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From the Department of Pharmacology University of Göteborg, Sweden

The Effect of Benzquinamide on the Metabolism of Catecholamines in Rabbit Brain

By

Jan Häggendal and Björn-Erik Roos

(Received January 27 1967)

Benzquinamide, a benzoquinolizine closely related to tetrabenazine, causes a mild sedation in rats and monkeys (WEISSMAN & FINGER 1962) and mice (CARLSSON & LINDQVIST 1966) and has marked effects on conditioned avoidance responses in the cat (WEISSMAN & FINGER 1962) After low doses of benzquinamide the alterations in conditioned avoidance responses are not accompanied by a diminution of the brain stores of noradrenaline (NA) or 5-hydroxytryptamine (5-HT) while larger doses cause some depletion of both amines (WEISSMAN & FINGER 1962). CARLSSON & LINDQVIST (1966) observed a decrease in dopamine (DA) and NA levels in the mouse brain following the intraperitoneal injection of 50–200 mg of benzquinamide. They also found that benzquinamide enhanced the effects of reserpine on behaviour and on brain catecholamine levels.

The present investigation was carried out in order to evaluate the effects of benzquinamide on brain amine levels and on catecholamine metabolism The levels of dopamine (DA) dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the corpus striatum, and the levels of NA normetanephrine (NM) and DA in whole brain were measured after the intravenous administration of benzquinamide to rabbits.

Methods

The experiments were performed on rabbits weighing about 1 5 kg which were kept at 23 The animals were killed with an intravenous injection f air at various intervals after the intravenous dministration of benzquinamide The brains were immediately removed and some of them put on an ice-chilled glassplate for dissection of the corpus striatum and the brain stem Amine analyses were performed by the following methods NA nd NM were determined according to HÄGGENDAL (1962, 1963a & b) nd DA according to CARLSSON & W LDECK (1958) with the modifications described by CARLSSON & LINDQVIST (1962). DOPAC and HVA were determined by methods of ANDÉN, ROOS & WERDINIUS (1963a & b).

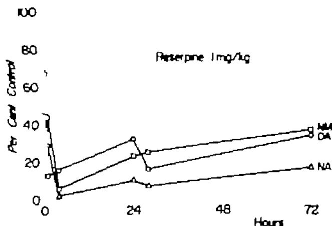


Fig. 1 The catecholamine levels in rabbit brain after reserpine (1 mg/kg i. v.) in per cent of normal levels. Single observations. Noradrenaline (NA). Normetanephrine (NM). Dopamine (DA)

Results

The effect of benzquinamide was compared with that of reserpine. The effect of reserpine was studied in the present investigation but data from previous studies were also used (ANDÉN ROOS & WERDINIUS 1963 a & b HÄGGENDAL 1963 b HÄGGENDAL, LINDQVIST & ROOS 1966)

The lowest levels of NA NM and DA were obtained four hours after administration of reserpine (1 mg/kg, i.v.) The levels then increased slowly (fig. 1 and 2)

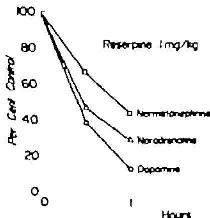


Fig. 2. The catecholamine levels in rabbit brain after reserpine (1 mg/kg i. v.) in per cent of normal levels. After 30 minutes mean values of three observations single observations after one hour

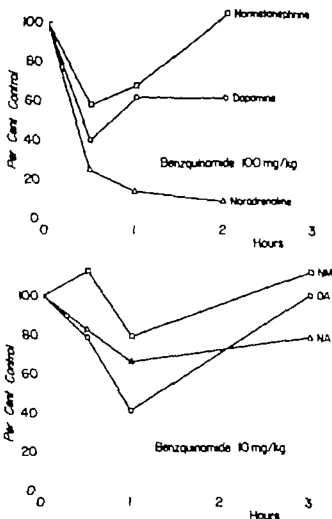


Fig. 3a and b. The catecholamine levels after benzquinamide 100 mg and 10 mg, respectively in per cent of normal levels. Mean values of two observations.

After benzquinamide (100 mg/kg) the decrease of these levels (fig. 3a and b) appeared to be rapid and this is also seen after reserpine (fig. 2), though, the recovery of NM levels was more rapid than after reserpine. A lower dose of benzquinamide (10 mg/kg) did not cause any significant change in the NM-levels. The NA-levels were decreased to a lesser extent than after 100 mg/kg of the drug. The DA levels were also decreased but the values returned to normal after 3 hours. The changes in the DA-levels of the corpus striatum followed the same pattern as that in the whole brain (fig. 4).

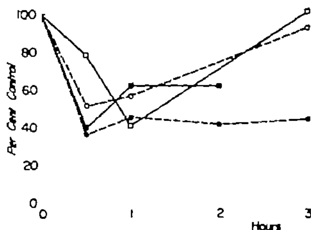


Fig. 4. Dopamine (DA) in per cent f normal values in whole brain and corpus striatum after benzquinamide 10 and 100 mg/kg, respectively
O———O corpus striatum after 10 mg/kg. ●———● corpus striatum after 100 mg/kg. □———□ whole brain after 10 mg/kg. ■———■ whole brain after 100 mg/kg.

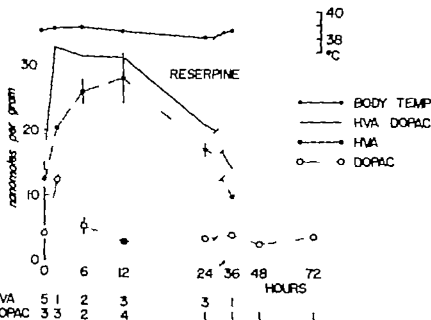


Fig 5. Concentration of DOPAC and HVA in rabbit corpus striatum at various intervals after a single i. injection of reserpine (1 mg/kg body weight). (From Andén, Roos and Werdinius, Life Sci. 1964 3, 149–158.)

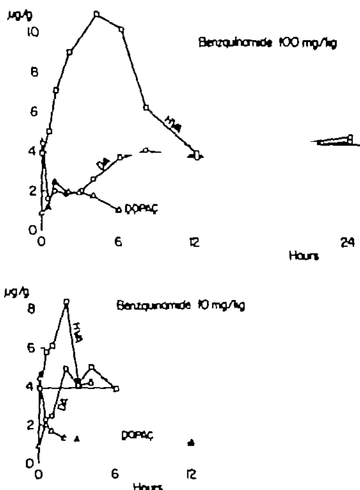


Fig. 6a and b. The levels of dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the corpus striatum after benzquinamide 10 and 100 mg/kg in per cent of normal levels. Mean values of two to four observations.

The levels of DOPAC and HVA are increased after reserpine as shown in fig. 5 (ANDÉN, ROOS & WERDINIUS 1964). There was a detectable rise in the levels of DOPAC and HVA as early as 30 min. after intravenous administration of benzquinamide (fig. 6a and b). The amounts of DOPAC and HVA were higher and remained in the brain longer after 100 mg/kg of benzquinamide than after 10 mg/kg. The level of HVA reached its maximum about four hours after the injection of 100 mg/kg while the DOPAC-level already seemed to reach its peak after one hour. After 10 mg/kg both maxima appeared somewhat earlier than after

100 mg/kg. After both doses the DOPAC values returned to normal levels while the HVA values were still elevated

One hour after the administration of 5 mg/kg of benzquinamide, the HVA levels increased to 7.5 μg/g tissue which was about twice the normal value (3.9 μg/g) while the DA values remained normal. One hour after 1 mg/kg of benquinamide both the NA the DA and the HVA values were normal.

Almost immediately after injection of benzquinamide (100 mg/kg) the animals were heavily sedated and showed decreased responses. The animals were most sedated during the first few hours. This picture was still easily detectable as long as four hours but absent six hours after the injection. Neither miosis nor ptosis were observed. No obvious changes in the behaviour could be detected after administration of 10 mg/kg or less of benzquinamide.

Discussion

The decrease in the NA and DA levels in brain after benzquinamide may be due either to one or both of the following factors inhibition of synthesis or release of the amines. The simultaneous increase of the acid metabolites is evidence against the former and favours the latter assumption. The same pattern occurs after reserpine (ANDÉN, ROOS & WERDINIUS 1964). However the effects of benzquinamide on monoamines seem to be of a shorter duration as compared with those produced by reserpine (WEISSMAN & FINGER 1962 CARLSSON & LINDQVIST 1966) This is in agreement with the present results which show that as early as 12 hours after a single injection of 100 mg/kg benzquinamide the levels of DA and HVA in the corpus striatum had returned to normal (fig. 6a). Furthermore, after 10 mg/kg these levels become normal more rapidly

With regard to the effect on behaviour benzquinamide had a short period of action even in high doses compared with reserpine. This is in agreement with the results of WEISSMAN & FINGER (1962) and CARLSSON & LINDQVIST (1966).

The decrease in the NM value seen after 100 mg/kg of benzquinamide is the only change in the amines observed that could possibly be correlated to the changed behaviour After 10 mg/kg the gross behaviour appeared to be normal and the NM level was within the normal range while the NA and DA levels were somewhat decreased

The results illustrate again that the levels of NA and DA in the tissues cannot be directly correlated to function. Similar phenomena have also been seen and discussed after administration of reserpine (e.g. EVERETT &

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WIEGAND 1962 HÄGGENDAL & LINDQVIST 1963 & 1964 CARLSSON 1965 SEDVALL & THORSSON 1965 HÄGGENDAL, LINDQVIST & ROOS 1966)

Normal nerve function probably requires only a very small amount of NA in the tissue Most of the NA normally present seems to be without immediate functional significance. It is possible that benzquinamide primarily affects this latter fraction while having only a short lasting action on the functionally important fraction. Thus one main difference between the two drugs may be that after reserpine the storage mechanism of most nerve granules is irreversibly blocked and that normal catecholamine levels in the nerve terminals are reached again by the downtransport of fresh granules formed in the cell bodies of the neurones (DAHLSTRÖM & HÄGGENDAL 1966) After benzquinamide, however the effect on the granules may be reversible like the effect of tetrabenazine on rat amine storage granules (DAHLSTRÖM 1966 HÄGGENDAL 1968). That the level of HVA is increased one hour after 5 mg/kg of benzquinamide while the DA level is almost unchanged might suggest an increase in the synthesis of the amines. This may be the result of a receptor blocking effect and the rapid onset of behavioural activity of the drug may support this view However other explanations are possible, e.g. decreased release from the functional important pool in the nerves.

There are some further observations which show differences between reserpine and benzquinamide. Signs of ptosis and miosis which are seen after reserpine administration were absent after benzquinamide. The effect of benzquinamide on the acid metabolites is more short lasting than that seen after reserpine. CARLSSON & LINDQVIST (1966) showed that tetrabenazine and prenylamine partially protect the animals against the depleting action of reserpine while benzquinamide enhanced this action. They suggest that the site of action of benzquinamide is different from that of reserpine.

The present investigation has shown that after benzquinamide there is a release of catecholamines which has several features in common with reserpine but that the two drugs may also have different sites of action.

Summary

Benzquinamide given to rabbits in doses of 100 or 10 mg/kg lowers the brain levels of NA and DA. Simultaneously the DOPAC and HVA values are increased. This argues in favor of a reserpine-like action of the drug and against an inhibition of the synthesis of the amines. After benzquinamide, both the behaviour of the animals and the production of amine metabolites are affected for a shorter period of time than after

reserpine treatment. A relatively constant concentration of DA together with an increase in the levels of HVA after only 5 mg/kg of benzquinamide may indicate that the drug also increases the synthesis of DA to some extent. Judging from the metabolic pattern produced, benzquinamide has several features in common with reserpine but also differs to some extent. This supports the view that the two drugs have different sites of action.

Acknowledgements

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Effect of Prenylamine on Locomotor Activity and Brain Monoamine Levels in Mice

By

Liisa Ahtee and Niilo Kärki

(Received October 12, 1967)

Prenylamine reduces the noradrenaline (NA) and 5-hydroxytryptamine (5HT) levels in the rat brain (Schöne & Lindner 1960). This substance is an amphetamine derivative and structurally different from Rauwolfia alkaloids and related compounds, the tranquillizing effect of which has been associated with their ability to decrease brain monoamine levels. These facts prompted us to investigate how the prenyl amine-induced changes in brain monoamines are related to its behavioural effects.

Material and Methods

Male albino mice weighing 18 to 21 g were used. During the experiments the room temperature was maintained at 22–24°. The animals were injected at the same time of the day, viz. 9–10 a.m., to avoid any interference by possible daily fluctuations in the NA and 5HT levels of the brain (M[illegible] & Patschke 1963). The drug, prenylamine, N-(3'-phenyl-propyl-(2'))-1,1-diphenyl-propyl-(3)-amin (Segontin ®, Farbenwerke Hoechst AG), was used as water soluble gluconate, and was administered subcutaneously. The doses are expressed in terms of bases. The control animals were injected with the vehicle only.

The spontaneous locomotor activity of mice was measured in a photoelectric motility box, made of black acryl, the size being 30 × 20 × 10 cm. The interruptions of the light beam were counted by an impulse counter. The animals, one at a time, were kept in the photocell box for two to five minutes. The locomotor activity was expressed as the number of interruptions per time unit.

The mice were killed by decapitation. The NA content of the brain was determined spectrophotofluorimetrically mainly according to the method of Shore & Olin (1958) and 5HT was determined by the method of Bogdanski *et al.* (1956) with minor modifications.

The arithmetical means and s.e.m. were calculated. The statistical significance of the differences between two means was determined by Student's t-test.

Table 1

Locomotor activity of mice at various intervals after subcutaneous administration of prenylamine (20 mg/kg and 100 mg/kg). Each animal was tested in the photocell cage for 2 min. at the given times. The figures given are the mean values of interruptions of light beam per 2 min. Standard error of the mean and the number of animals in each group are given in the table.

Hours after injection	Locomotor activity (counts/2 min.)		
	Control	Prenylamine 20 mg/kg	Prenylamine 100 mg/kg
¼	36.6 ± 2.2	36.1 ± 3.6	29.3 ± 2.1
½	31.5 ± 2.1	19.4 ± 2.9	10.7 ± 2.2[1]
1	27.8 ± 2.7	9.8 ± 2.3[1]	8.0 ± 1.7[1]
1½	17.9 ± 2.1	5.1 ± 1.4[1]	6.6 ± 1.4[1]
2	15.5 ± 2.0	5.7 ± 1.8[1]	6.1 ± 1.2[1]
3	11.0 ± 1.6	3.4 ± 1.1[1]	2.6 ± 0.6[1]
4	14.2 ± 2.3	8.1 ± 2.5	2.7 ± 1.0[1]
5	8.8 ± 1.7	6.2 ± 1.7	3.5 ± 1.0
Number of animals	50	20	23

[1]) Change is highly significant compared to control ($p < 0.001$)

Results

Effect of prenylamine on motility

In preliminary experiments (table 1) the spontaneous locomotor activity of mice was measured at various intervals during 5 hrs. after administration of 20 and 100 mg/kg of prenylamine. In this series of experiments the same mouse was placed 8 times in the photocell box for motility testing. The motility of control mice was progressively and statistically significantly reduced during the experiment from 37 to 9 counts/2 min. However the reduction of motility was more marked in the prenylamine-treated mice and the higher dose seemed to be more effective. Statistically significant changes were recorded, in the mice given 20 mg/kg of prenylamine, 1 to 3 hrs. after the injection ($p < 0.001$) and in the mice given 100 mg/kg of prenylamine, ½ to 4 hrs. after the injection ($p < 0.001$).

Effect of prenylamine on the NA and 5HT level of the brain

Figure 1 shows the effect of prenylamine on the NA and 5HT contents of the brain 5 hrs. after s.c. prenylamine administration. Of the brain amines NA showed the highest decrease. There already was an 18%

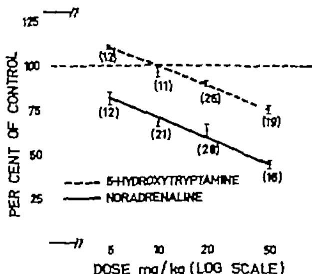


Fig. 1 Effect of different doses of prenylamine on the oradrenaline and 5-hydroxytrypt amine levels of mouse brain. The mice were killed 5 hours after the subcutaneous injection of the drug. The vertical bars refer to the standard errors of the mean and the umber f determinations is given in brackets.

depletion of NA with 5 mg/kg of prenylamine ($p < 0.001$) Approximately 4 to 6 times more prenylamine was needed to cause similar re ductions in the brain 5HT level as in the brain NA level. The dose of 10 mg/kg prenylamine caused a 32 / depletion of brain NA ($p < 0.001$), although this dose of prenylamine did not affect the 5HT level. 20 mg/kg of prenylamine significantly reduced the brain 5HT level ($p < 0.001$). The highest used dose of prenylamine (50 mg/kg) reduced the brain NA by 56 / and the brain 5HT by 25 /.

In this series of experiments the spontaneous motility of mice was also measured (table 2) The same animal was placed only once in the photocell cage and the average number of interruptions of the lightbeam remained unchanged in the control groups. The dose of 5 mg/kg of prenyl amine reduced the motility only slightly

Statistically significant ($p < 0.001$) reductions in the motility of the mice were observed after 10 mg/kg of prenylamine, and the higher the dose the more pronounced was the sedative effect.

Brain monoamine levels and motility after prenylamine administration

In order to acquire more information on the possible correlation between prenylamine-induced sedation and brain monoamine depletion,

Table 2

Locomotor activity f mice at various intervals after subcutaneous administration of prenylamine. Each animal was tested in the photocell cage only once. The figures given are the mean values of interruptions of lightbeam per 2 min. Standard error of the mean and number of animals in each series are given in the table.

Dose mg/kg	Number f	Locomotor activity (counts/2 min.) ½	1½	3 hr	5 hr
Control	99	46.3 ± 1.2	46.3 ± 1 6	45.2 ± 1 3	46.4 ± 1 9
5	27	39 7 ± 2.2	41 0 ± 3 0	40.9 ± 2.4	41.0 ± 2.3
10	18	33 8 ± 2.8	31.2 ± 2.9	38.3 ± 3 6[1])	43 9 ± 3 4[1])
20	36	27 6 ± 1 8[1])	29 5 ± 1.5[1])	32.3 ± 1 8[1])	34.9 ± 2.1[1])
50	18	28 7 ± 3 1[1])	21 1 ± 2.3[1])	26 7 ± 2.4[1])	26.3 ± 2.6[1])

[1]) Change is highly significant compared t control ($p < 0\ 001$)

an additional series of experiments was performed. The spontaneous activity of mice was recorded ½ ½ 1½ 3 5 or 10 hrs. after prenylamine administration. Immediately after motility testing (5 min.) the mice were killed and their brain NA and 5HT concentrations determined. Figure 2 shows the results of these experiments. The changes in motility and brain monoamines are expressed as per cent of the control. The control mice were injected and tested in parallel with the prenylamine-treated mice (table 3).

Doses of 10 to 50 mg/kg prenylamine clearly reduced the motility Sedation began within ½ hrs. and reached its maximum with all doses used within 1½ hrs. The motility was reduced to 57 / of the control by 10 mg/kg and to 40 / of the control by the 50 mg/kg of prenylamine. Normal motility was recovered within 10 hrs. after 20 mg/kg of the drug. In animals treated with 50 mg/kg of prenylamine some sedation was observed even 10 hrs. after drug administration.

With all doses studied, prenylamine lowered the NA levels more than the 5HT levels. In the dose of 10 mg/kg of body weight, prenylamine lowered the brain NA level to 73 / of the control in 1½ hrs. and caused a maximum decrease (62 / of control) within 3 hrs. The higher doses of prenylamine (20 and 50 mg/kg) also lowered the brain NA usually in 3 hrs. However their effects appeared earlier 15 to 30 min. after administration of the drug, and were more marked i.e. 60 / and 47 / of the control respectively In addition the brain NA levels in mice treated with 50 mg/kg of prenylamine were still 72 / of control ($p < 0.05$) 10 hrs. after drug administration.

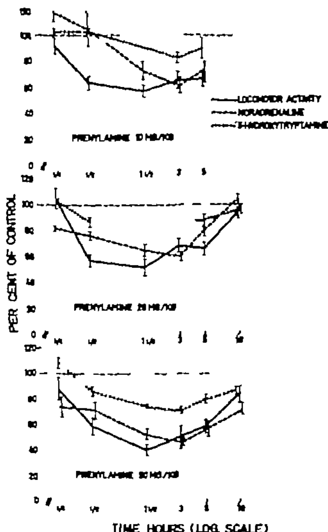


Fig. 2. Effects of different doses f prenylamine on the locomotor activity and brain noradrenaline and 5-hydroxytryptamine content f mice t various intervals after subcuta neous prenylamine administration. The locomotor activity was recorded for 5 min. and each animal was tested only once, after which it was killed and the brain monoamines determined. The values are given as percentages f control animals tested and killed at the same time of day (see table 3). Vertical bars refer to the standard errors of mean groups of 18 to 3. mice.

The prenylamine-induced decrease in 5HT concentration appeared more slowly and was less marked than the decrease in NA. The maximum decrease occurred usually in 3 hrs. At this time 10 mg/kg of prenylamine depleted the brain 5HT to 83 / and 50 mg/kg to 71 / of the control,

Table 3

Locomotor activity and brain noradrenaline and 5-hydroxytryptamine content of control mice in the experiment described in fig. 2. Means ± s.e.m.

Hours after injection	Locomotor activity (counts/5 min	Brain noradrenaline (μg/g)	Brain 5-hydroxytryptamine (μg/g)
¼	54.3 ± 4.6	0.62 ± 0.07	0.63 ± 0.02
½	77.9 ± 5.9	0.63 ± 0.03	0.65 ± 0.02
1½	71.3 ± 4.0	0.65 ± 0.05	0.67 ± 0.02
3	64.0 ± 4.0	0.68 ± 0.05	0.67 ± 0.02
5	66.8 ± 3.2	0.65 ± 0.02	0.69 ± 0.02
10	74.8 ± 4.1	0.62 ± 0.05	0.65 ± 0.02

respectively. After the highest dose used (50 mg/kg), the brain 5HT was still significantly ($p < 0.01$) below the control level 10 hrs. after prenylamine administration.

Discussion

Prenylamine is structurally an amphetamine derivative in which there is a phenylisopropyl group attached to the amine group. In the study of the structure and action of phenylisopropyl derivatives VAN DER SCHOOT *et al.* (1962) found that prenylamine was ineffective in increasing the spontaneous motor activity of mice. However they did not pay attention to the motility decreasing effect which is clearly demonstrated in our experiments. As low a dose as 10 mg/kg of prenylamine administered subcutaneously significantly reduced the spontaneous locomotor activity of mice. LINDNER (1960) reported that the motility of rats was not affected by doses lower than 50 mg/kg of prenylamine.

An interesting observation made in our experiments was the association between the prenylamine induced sedation and the decreases in brain monoamines levels. Although the decrease in brain amines was greatest in NA, we also observed a fall in brain 5HT content even after relatively low doses of prenylamine (20 mg/kg). JUORIO & VOGT (1965) did not find any changes in brain 5HT concentration in rats although SCHÖNE & LINDNER (1960) reported that high (100 mg/kg) doses of prenylamine induced reduction also in brain 5HT. WERDINIUS (1967) too observed a correlation between the sedation and maximal effects on brain monoamines. In rabbits, dopamine in the corpus striatum and 5HT in the

anterior brain stem were significantly lowered by 5 mg/kg of prenylamine given intravenously (WERDINIUS 1967).

There is uncertainty about the role of monoamines as transmitters in the brain and also about the correlation between the brain monoamine levels and behaviour. Moreover there has been discussion about which of the monoamines if any is associated with the tranquillizing action of drugs (SULSER & BRODIE 1960, BRODIE *et al.* 1960). It is well-known that the tranquillizing Rauwolfia alkaloids also cause depletion of both brain NA and 5HT. The same is true of some other drugs e.g. benzoquinolizines (PLETSCHER *et al.* 1959). There are, however, drugs which are known to lower brain NA without affecting brain 5HT, e.g. Su 5171 (BRODIE *et al.* 1960) and certain dihydroxyphenylalanine-analogues. These drugs are reported to be non-sedative and therefore BRODIE *et al.* (1960) came to the conclusion that depletion of 5HT would be responsible for the sedative effect of reserpine. On the other hand, there are many studies in which a positive correlation between brain catecholamines and behaviour is obvious. CARLSSON *et al.* (1957) and BLASCHKO & CHRUŚCIEL (1960) have established that a catecholamine precursor, dihydroxyphenylalanine, causes significant increase of motor activity both in normal and reserpine treated mice.

Several studies indicate that there is a close similarity in the mechanism of action of prenylamine and of Rauwolfia alkaloids (CARLSSON & LINDQVIST 1966, EULER *et al.* 1964, LUNDBORG 1966). In earlier studies (KÄRKI & PAASONEN 1959) on the effect of a Rauwolfia alkaloid, Raunescin, we came to the conclusion that changes in brain NA content were better related to the sedative effect, than the changes in brain 5HT levels. PLETSCHER *et al.* (1959) have expressed similar opinions after experiments with certain benzoquinolizine derivatives. The present experiments also support this view since prenylamine seemed to induce sedation in doses which lowered only brain NA without affecting 5HT. It is however possible that changes in the intracellular distribution of monoamines which may possibly occur may be of more importance for drug action than the change in total monoamine content.

Summary

The changes in brain noradrenaline and 5-hydroxytryptamine induced by prenylamine were compared with its effects on locomotor activity in mice. In doses of 10 to 50 mg/kg injected subcutaneously prenylamine significantly reduced the motility of mice. Sedation began within 0.5 hr and reached its maximum within 1.5 hrs. Of the brain amines the nor

adrenaline level was decreased most Approximately 4 to 6 times higher doses of prenylamine were needed to cause a similar reduction in 5-hydroxytryptamine. Normal motility and brain amine levels were recovered within 10 hrs. after administration of lower doses of prenylamine. In mice treated with 50 mg/kg of prenylamine some sedation was still observed even 10 hrs. after drug administration. In addition the brain noradrenaline was depleted by 28% and 5-hydroxytryptamine by 14%. These findings suggest that the prenylamine-induced sedation is correlated with the depletion of brain monoamines and particularly with that of noradrenaline.

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Hexobarbital (Enhexymalum NFN) Sleeping Times and EEG Threshold Doses as Measurements of Tolerance to Barbiturates in the Rat

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Tolerance develops to many pharmacological agents. It is easily defined as a decreasing response on repeated administration of the same dose of a drug or a need to increase the dose in order to obtain the original response. Tolerance can develop to the central nervous system (CNS) depressants but they can also induce a state called physical dependence. SEEVERS & DENEAU (1963) have defined physical dependence as "The state of latent hyperexcitability which develops in the cells of the central nervous system of higher mammals following frequent and prolonged administration of the morphine like analgesics alcohol barbiturates and other depressants" The CNS depressants have in this respect been divided into two different groups 1) The morphine-like substances, and 2) Sedative hypnotics. Included in the second group are alcohol and barbiturates (SEEVERS & DENEAU 1963). Most of the work has been concerned with the first group (COLLIER 1966 DENEAU & SEEVERS 1964 SEEVERS & DENEAU 1963) and extensive studies on the sedative-hypnotics are still lacking (DENEAU & SEEVERS 1964)

In the present paper a rapid anesthesia threshold method (WAHLSTRÖM 1966a) has been used to study changes induced by short-term treatment with pentobarbital and long term treatments with barbital. A preliminary report on the effects of long-term alcohol treatment has previously been given (WAHLSTRÖM 1966c).

Methods

Male Sprague-Dawley rats (Anticimex) were used. The animals weighed around 300 g before the experiments and around 400 g at the end of the long-term experiments (results part B). The rats were kept in thermostatically controlled room at 30 The light in the

room had a cycle of 12 hours of light and 12 hours of darkness. The light was turned on at 8 a.m. The rats had access to water and food pellets ad libitum. All experiments were performed during the light hours in the rat room.

In the threshold determinations racemic hexobarbital (enhexymalum NFN) sodium was infused into the tail vein at constant rate. Racemic hexobarbital sodium (evipan ®) was obtained as a powder in a commercial preparation from Bayer AG. A stock solution (to be used within an hour of preparation) with a concentration of 100 mg/ml was prepared with distilled water. From this solution was taken the amount needed to give each rat a dose of 150 mg/kg. This amount was further diluted with 0.25 % sodium chloride to final volume of 1 ml. The rate of the hexobarbital infusion was 0.25 mg/kg/sec and the volume rate was thus 0.1 ml/min. The electroencephalogram was recorded during the infusion on Schwarzer electroencephalograph. As the end point the first burst suppression of 1 second or more was taken (the silent second). The threshold used was the dose of hexobarbital needed to btain this silent second. Details of the threshold determinations have previously been given (WAHLSTRÖM 1966 b).

When the silent second had occurred the infusion was stopped. The amount f hexobarbital infused after the silent second depended on whether the first silent second in the EEG-curve was actually observed during the experiment or only later on. The ensuing sleeping times (the times without righting reflex) were measured in the rat room with an automatic device consisting of recording beds. All sleeping times after threshold determinations, where more than 7.5 mg/kg of hexobarbital sodium had been infused, in addition to the dose needed for the silent second were discarded. For details regarding the sleeping time measurements, see WAHLSTRÖM (1966 b).

In some experiments sleeping times were similarly recorded but after fixed doses of barbiturate. These barbiturates were given intraperitoneally in the lower left part of the abdomen. Hexobarbital sodium was used as the stock solution (100 mg/ml) described bove. Pentobarbital (mebumalum NFN) sodium was given in solution containing 30 mg/ml or 40 mg/ml and barbital (diemalum NFN) sodium in solution containing 40 mg/ml. All solutions were prepared immediately before use with distilled water.

All control groups received 0.9 % sodium chloride instead of the barbiturate. All doses in the following have been given as th sodium salt.

Results

A Tolerance development after treatments with pentobarbital

In two series of experiments intraperitoneal injections of pentobarbital (30 mg/kg and 40 mg/kg respectively) were performed once a day for four days. In one group (5 rats) in each series, tolerance was tested approximately 24 hours after the last treatment by threshold determinations (Th-test) with hexobarbital and in another group (9–10 rats) it was tested by sleeping time measurements after a fixed dose of 150 mg/kg hexobarbital given intraperitoneally (S-test). No change in threshold dose or hexobarbital sleeping time was recorded in these series.

In the next three series tolerance was similarly tested but the schedule of pentobarbital treatments was more intense and is shown in fig. 1 Before the two types of tests (S and Th) with hexobarbital performed on

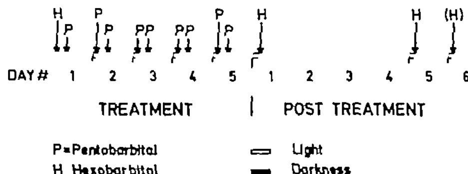


Fig. 1 Treatment schedules used in series III, IV and V The arrows indicate approximate time of treatment. Long arrows indicate experimental recording of sleeping times with pentobarbital and silent second threshold or sleeping times with hex barbital. The dose of pentobarbital was 30 mg/kg. For further explanation see text.

treatment day 1 another test had been performed four days earlier which was discarded This discarded test was the first one ever done on these animals. Pentobarbital was administered in a dose of 30 mg/kg starting in the afternoon after the second test and was then administered twice daily The development of tolerance was tested 16–21 hours after the last treatment. Five (in the last series six) days after the end of the pentobarbital treatment, another test was performed

In table 1 the sleeping times after the second and eighth pentobarbital treatments are shown for the groups which, before and after treatment, were given S or Th-tests. It is evident from table 1 that the two groups had

Table 1

Sleeping times in minutes after the second and the eighth treatment with 30 mg/kg pentobarbital i. p.

Series	n	Group later given S-test: second treatment Mean ± s.e.m.	Group later given S-test: eighth treatment Mean ± s.e.m.	n	Group later given Th-test: second treatment Mean ± s.e.m.	Group later given Th-test: eighth treatment Mean ± s.e.m.
III	7	45.5 ± 8.7	30.5 ± 2.1	5	36.2 ± 4.1	28.6 ± 2.0
IV	9	34.8 ± 4.8	28.4 ± 3.7	5	42.5 ± 4.1	28.4 ± 1.8
V	8	31.1 ± 4.9	26.6 ± 3.2	4	32.5 ± 3.2	31.5 ± 5.2
Total	24	36.7 ± 3.6	28.5 ± 1.7	14	37.4 ± 2.4	29.4 ± 1.6

Table 2

Sleeping time in minutes after 150 mg/kg hexobarbital i p. (S-test)

Series	n[1]	Before pentobarbital treatment	16–21 hours after pentobarbital treatment	5–6 days after pentobarbital treatment
		Mean ± s.e.m.	Mean ± s.e.m.	Mean ± s.e.m.
III	8	64.9 ± 9 7	37.5 ± 2.5	49.5 ± 6.1
IV	8	54 9 ± 11 7	40.2 ± 2.8	44.6 ± 3 7
V	8	54.6 ± 8.8	34.5 ± 1.0	40.6 ± 3 1
Total	24	58.1 ± 5.7	37 4 ± 1.3	44 9 ± 2.6

[1]) For technical reasons three rats included in table 1 had to be excluded here, but three rats which were excluded from table 1 could be used here instead.

similar sleeping times and that there was a reduction in sleeping time between treatment two and treatment eight in both groups A clear tolerance had thus developed measured as pentobarbital sleeping times during the treatment.

The tolerance measured as hexobarbital sleeping times (S-test) after a fixed dose of 150 mg/kg is shown in table 2. Compared with the pre-experimental values the sleeping times 16–21 hours after the end of the pentobarbital treatment were reduced by approximately 35%. A clear reduction was also evident 5–6 days after the end of treatment. With

Table 3

Threshold doses of hexobarbital needed to obtain the silent second (Th-test)

Series	n	Threshold dose mg/kg: Before pentobarbital treatment	16–21 hours after last pentobarbital dose	5–6 days after last pentobarbital dose	Dose at 16–21 hours in % of dose before pentobarbital treatment
		Mean ± s.e.m.	Mean ± s.e.m.	Mean ± s.e.m.	Mean ± s.e.m.
III	5	69 6 ± 4.1	70.5 ± 3.8	62.4 ± 3 9	103.0
IV	6[1])	63 6 ± 3.6	68.3 ± 1 8	70.5 ± 3.8	108.9
V	4	66.5 ± 0.2	71.4 ± 4.1	76.7 (n = 3)	108.5
Total	15	66.3 ± 2.0	69 9 ± 1 7	69.0 ± 3 6 (n = 14)	106.8 ± 4.2

[1]) Due to technical reasons one rat included here had to be excluded from table 1

Table 4

Sleeping times in minutes after threshold doses of hexobarbital needed to obtain the silent second.

Series	n	Before pentobarbital treatment	16–21 hours after pentobarbital treatment	5–6 days after pentobarbital treatment
		Mean ± s.e.m	Mean ± s.e.m.	Mean ± s.e.m.
III	5	18.6 ± 2.1	16.0 ± 1.4	15.8 ± 1.2
IV	6[1])	19.6 ± 2.9	18.7 ± 0.7	19.3 ± 1.9
V	4	27.8 ± 5.3	17.8 ± 2.1	21.1 (n = 3)
Total	15	21.4 ± 2.1	17.5 ± 0.8	18.5 ± 1.2 (n = 14)

1) Due to technical reasons one rat included here had to be excluded from table 1

regard to sleeping times, there is thus a clear cross-tolerance between pentobarbital and hexobarbital.

The results of the threshold determinations (Th-test) performed in the groups are shown in table 3. There was no difference between the threshold doses needed before and after pentobarbital treatment. No cross tolerance could thus be demonstrated by this test.

In table 4 the sleeping times after the threshold doses given in table 3 are shown. These sleeping times have a pattern of changes similar to those obtained after a fixed dose of hexobarbital of 150 mg/kg intraperitoneally (table 2). None of the differences were however significant (Student's t test, calculated on the individual differences). The reduced standard error in sleeping time in the 16–21 hour test is due to the reduced number of rats with long sleeping times. None of the rats at 16–21 hours had sleeping times longer than 21.4 minutes as compared with 8 in the *pre-experimental sleeping time determinations. The fairly short sleeping* times obtained after threshold doses of hexobarbital are thus only slightly influenced by the changes induced by the pentobarbital treatment.

B Tolerance development after treatments with barbital

Sodium barbital or NaCl was administered once daily for 25 days and the Th-test carried out every second or third day after the end of this treatment. The first test was performed 24–27 hours after the ... ital administration. The results of this series are shown in fig. 2 ... that the hexobarbital threshold had increased (by ...

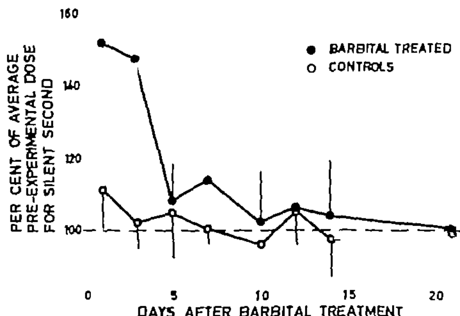


Fig. 2. Changes in hexobarbital thresholds after treatment with barbital 200 mg/kg i.p. every day for 25 days. The average pre-experimental threshold dose for the controls was 63.6 mg/kg and for the barbital treated animals 63.5 mg/kg. These averages were based on the mean of 8–10 determinations in each rat. The average weights of the animals were 344 g and 355 g before starting the treatment with barbital and sodium chloride respectively. The corresponding weights on the first day after treatment were 364 g and 363 g. The bars plotted in only one direction indicate twice the s.e.m. The number of animals was 4 except in the points without bars where it was 3

on the day following the end of the barbital treatment. Two days later the threshold was still increased. 5 days after the end of the barbital treatment the tresholds were about the same as in the controls, but still slightly increased as compared with the base line. In this experiment a marked tolerance to hexobarbital in the Th test had thus been induced by the barbital treatment.

The increase in threshold after long-term barbital treatment seemed to be of maximal absolute magnitude on the first day (with days as the time scale) after cessation of treatment. The changes in threshold could therefore be followed during the barbital treatment, if the barbital administration was delayed on the test day until after the threshold determination. In the following two series (XI and XII) the changes in threshold were thus tested every week during barbital treatment.

The pre-experimental values of silent second and sleeping time for the

Table 5

Pre-experimental values of threshold dose and corresponding sleeping times.

Series	Treatment to be given	Dose for silent second mg/kg			Sleeping time min.		
		Mean	s.e.m.	n	Mean	s.e.m.	n
XI[1]	Barbital	65.2	1.6	16	19.8	1.3	16[2]
	N Cl	60.4	1.5	12	18.1	1.2	11[2]
	Total	63.1	1.2	28	19.1	0.9	27
XII[3]	Barbital	67.0	1.6	11	26.5	1.3	11
	N Cl	64.1	2.4	6	25.5	1.5	6
	Total	66.0	1.3	17	26.2	1.0	17

[1]) One rat had no acceptable pre-experimental value for sleeping time. Three had one pre-experimental value.

[2]) Seven rats had one pre-experimental value.

[3]) The differences between series XI and XII in dose for silent second had $p > 0.05$ and for sleeping times a $p < 0.001$ (Student's t test).

rats participating in the two series are shown in table 5. For each rat the average pre-experimental threshold dose was calculated on two determinations in series XI and on 3 determinations in series XII. The number of sleeping time values for each rat varied depending on whether the infusion was stopped or not before 7.5 mg/kg had been infused after the silent second (see methods). One rat in series XI had no acceptable sleeping time and had to be excluded from the sleeping time data. Table 5 shows that there was no detectable difference between the two series with regard to threshold dose but a difference with regard to the subsequent sleeping time.

Two rats died in series XI and 3 in series XII during the barbital treatment (200 mg/kg i.p. every day). Four rats died among the controls of series XII. All these rats have been excluded.

The sleeping times after the barbital dose were measured on 5 occasions with the automatic beds. When there is a very gradual return of the righting reflex, as is the case after barbital administration, the beds tend to give lower values for the sleeping time than direct observations, but have approximately the same error (WAHLSTRÖM 1966b). The recorded sleeping times after barbital 200 mg/kg are given in fig. 3. There was no clearcut trend in barbital sleeping time during the treatment. For unknown reasons, the sleeping times were shorter in series XI than in series XII.

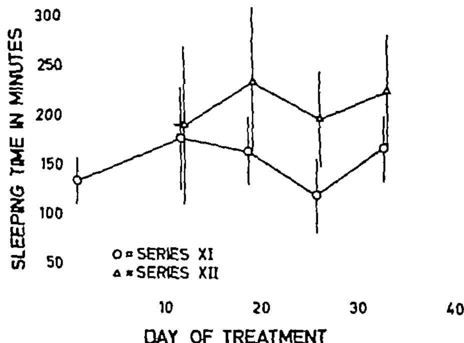


Fig. 3 Sleeping times after barbital (200 mg/kg i.p.) during the treatments in series XI and XII. The number of animals in each point in series XI were 14 13 10 9 14 and in series XII 11 10, 10, 10, 10. The bars indicate twice the s.e.m. Due to the gradual disapperance of the righting reflex sleeping times shorter than 10 min. were excluded.

Also after threshold doses of hexobarbital shorter pre-experimental sleeping times were obtained in series XI (table 5). There was however no relation between the pre-experimental hexobarbital threshold dose and the barbital sleeping time on treatment day one (correlation coefficient r = 0 01 DF = 23)

During the barbital treatment weekly Th tests were performed 24–34 hours after the last barbital injection. On every test occasion, series XI was tested on an average 3 hours earlier after the barbital than series XII. When the animals had recovered from the threshold doses of hexobarbital the new dose of barbital was administered. The first two doses of barbital after a hexobarbital threshold determination were thus only separated by 14–16 hours instead of the 24 hours.

Fig. 4 shows that there was a gradual increase in threshold during the treatment. The maximal increase (after 5 weeks) was approximately the same as in the series treated for 25 days (fig. 2). The most rapid increase occurred in the first three weeks of treatment but it cannot definitely be

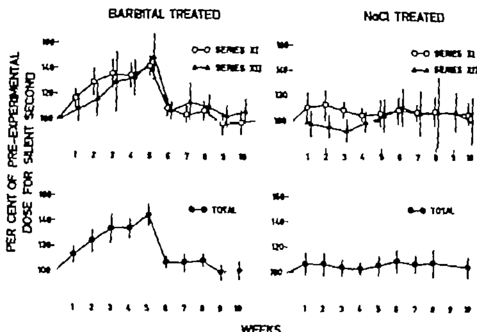


Fig. 4 Changes in hexobarbital threshold during and after daily treatment for 5 weeks with barbital 200 mg/kg i.p. The treatment is indicated by the filled bar along the x-axis. The bars at each point indicate twice the s.e.m. The pre-experimental values for silent second threshold are given in table 5. The average weights in series XI before the treatment were 331 g and 336 g and after 5 weeks of treatment 366 g and 363 g in the controls and barbital treated groups. The corresponding figures in series XII before the treatment were 361 g and 393 g and after treatment 387 g and 399 g. The number of animals in each point in the barbital treated animals in series XI were 12–16, in series XII 7–11 (total 19–27) and in the NaCl treated animals in series XI 10–12, in series XII 4–6 (total 15–18). The smallest number occurred in weeks 9 and 10.

stated whether the maximal possible increase had been reached after treatment with barbital for 5 weeks. There was no large difference between the two series although the increase in threshold was somewhat more linear in series XII. One week after the last barbital treatment, the threshold had dropped and only a slight elevation (approximately 10 %) remained. There was, however, an increase of the same magnitude in the controls. Four to five weeks after the end of the treatment the threshold had returned to the pre-experimental level.

Is there a correlation between the individual sensitivity to the anaesthetic activity of barbital and the threshold elevation resulting from repeated barbital doses? Due to the slow elimination of barbital the only barbital sleeping time which can be used for the correlation is the first one. The correlations between this barbital sleeping time and the absolute in-

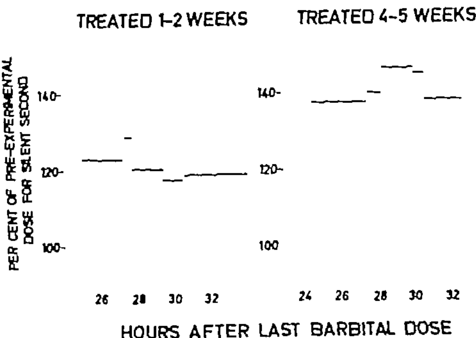


Fig. 5. The relationship between the time after the last barbital dose and the median threshold dose in series XI and XII. The barbital treated animals in fig. 4 have been used. The values obtained after treatment for 1 and 2 weeks and after treatment for 4 and 5 weeks have been pooled. Each class consists of 10 animals except the last one where there were 9 animals after 4 and 5 weeks. For further explanation see text.

crease in hexobarbital threshold dose after 2, 3 4 and 5 weeks of barbital treatment were all positive with r between 0.12 and 0.51 Only the largest one, however had a P value less than 0 05 (DF = 17). Thus, probably due to the limited amount of data, only a doubtful correlation between initial barbital sleeping time and subsequent increase in threshold dose could be established. The correlations between the absolute increases in threshold dose in the same rat after various periods of barbital treatments were also all positive (r between 0.12 and 0.69 DF = 17). In addition, in this case only one coefficient (between the increase in the third and the fifth week) had a P value less than 0.01 Thus the presence of a characteristic response in the individual rat during the barbital treatment could not be proved, even though it is probable.

It seemed probable that the interval between the last barbital injection and the threshold determination had some effect. The time interval in which the data were collected was between 24 and 34 hours after the last barbital injection. The data obtained after one and two weeks of barbital

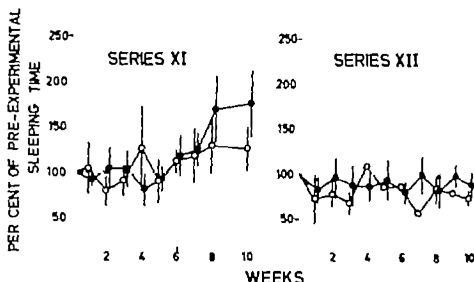


Fig. 6. Sleeping times after threshold doses of hexobarbital during and after daily treatments with barbital 200 mg/kg. The treatment is indicated by the filled bar along the x-axis. Filled circles denote barbital-treated and unfilled circle denote controls. The bars at each point indicate twice the s.e.m. In the points with n less than five no s.e.m. is plotted. The pre-experimental values are given in table 5 The same animals as in fig. 4 were used but some had to be excluded as too much hexobarbital was infused after the silent second. The number of animals in each point were in series XI barbital treated 9–15 and NaCl treated 7 11 and in series XII barbital treated 6–10 and NaCl treated 2–6.

treatment and after four and five weeks were pooled into two groups of 50 and 49 observations respectively These pooled data were arranged with regard to the duration of the interval between the last barbital dose and the threshold determination The 10 experiments with the shortest interval were then made into a first class, the 10 following intervals into a second and so on. Fig. 5 shows the results. There does not seem to be any marked non random change in the thresholds in the time interval used for the threshold determinations.

As the two series of experiments had different pre-experimental sleeping times after the silent-second dose (table 5), the values for hexobarbital sleeping times are only given separately for the two series and have not been treated together The results are shown in fig. 6 During the period of daily barbital treatment there was no change in sleeping times after a threshold dose of hexobarbital despite the fact that these doses had been increased

After the end of the barbital treatment a puzzling observation was made in series XI There was an increase in hexobarbital sleeping time

Table 6

Sleeping times in minutes after threshold doses of hexobarbital.

Series	Treatment	Pre-exp.	Weeks after end of treatment					
			3			5		
			Mean	n	s.e.m.	Mean	n	s.e.m
XI	Barbital	19.8	30.9	12	2.3	35.5	12	3.5
XII	Barbital	26.5	22.8	9	3.2	22.4	7	1 6
XI	N Cl	18.1	25.6	9	2.6	27.0	11	2.1
XII	NaCl	25.5	21 0	5	1.9	19 7	5	1 7

Pre-exp. = Pre-experimental value from table 5.

after the threshold dose. No comparable change was seen in series XII As the pre-experimental averages in sleeping time in the two series (table 5) were different the absolute values are given in table 6.

No sleeping time values are given at 9 weeks in series XI (fig. 6). On this occasion all the rats received doses of hexobarbital which were individually identical with those given after 5 weeks of barbital treatment when tolerance was at a maximum. The appearance of a silent second was thus disregarded. No rats died. It was thus not possible to establish in this way whether the lethal dose had increased or not during the barbital treatment.

Discussion

A reduction of sleeping times after consecutive barbiturate injections is a simple and common way of demonstrating tolerance to barbiturates (see for instance GRUBER & KEYSER 1946 and MOIR 1937). In the present experiments such a reduction was obtained with pentobarbital in male rats (table 1). The hexobarbital sleeping times were also reduced after pentobarbital treatment (table 2), and this phenomenon is usually referred to as cross-tolerance. The existence of such a cross-tolerance between several barbiturates has been previously demonstrated by for instance GRUBER & KEYSER (1946).

This sleeping time tolerance to barbiturates depends to a very large extent on an induced increase in metabolism of the barbiturate (REMMER 1962, CONNEY & BURNS 1962). The induced increase in metabolism is not specific for the inducing barbiturate (REMMER 1962 CONNEY & BURNS

1962) It can thus, in addition often explain the cross-tolerance seen between barbiturates. The reason for the cross-tolerance with regard to sleeping-time between hexobarbital and pentobarbital seen in the present experiments without doubt largely depends on such an increased metabolism, as a metabolic cross-tolerance between pentobarbital and hexobarbital has been shown to exist in the rat (REMMER 1962).

The threshold determination was developed so as to be performed within such a short time (4-6 minutes) that interference from the metabolic degradation of hexobarbital could be disregarded That this assumption seems to be correct can be seen in series III IV and V (table 2 and 3). After the pentobarbital treatment the hexobarbital sleeping times (after 150 mg/kg i p) were reduced but no similar changes in silent second thresholds were obtained A faster rate of hexobarbital metabolism seen as a decreased sleeping time thus does not substantially influence the silent second threshold

Threshold determinations can be performed every second or third day without inducing changes in the thresholds as can be seen from the controls in fig. 2. Previous experiments have given similar results (WAHLSTRÖM 1966a) It is also unlikely that threshold determinations (doses around 60 mg/kg) every second or third day will induce changes in hexobarbital metabolism, since doses of 200 mg/kg once a day had no effect on sleeping times or on the metabolizing capacity in male rats (REMMER 1964) The determination of the silent second as such thus does not seem to have any lasting effects on the animal and repeated determinations on the same animal can be safely performed for following changes induced by other means.

That a change is induced in the CNS by long term treatments with barbiturates is clearly demonstrated by the convulsions which occur following abrupt withdrawal This has, for instance, been shown with regard to barbital in the cat (ESSIG & FLANARY 1959 & 1961)

Barbital as such could also be used in the usual experiments with sleeping times to study the CNS tolerance. The reason for this is that only a few per cent (approximately 5) of barbital is metabolized in for instance the rat (GOLDSCHMIDT & WEHR 1957 EBERT *et al* 1964 BURNS *et al* 1957) and this figure does not seem to be much influenced (a few per cent) by prolonged treatments (EBERT *et al* 1964). A drawback is the slow elimination of barbital and an ensuing cumulation that tends to mask a decrease in CNS sensitivity The maximal cumulation is well established within a week of daily treatments with barbital in the rat, as most of the barbital after a single administration has disappeared within four days (95%/75 hours (GOLDSCHMIDT & WEHR 1957) and 98 %/3–4 days (BURNS

et al 1957). In the present experiments no tolerance was, however seen in barbital sleeping times (fig. 3). Similar results have been obtained by EBERT *et al.* (1964) when a challenging dose of barbital (200 mg/kg) was given 24 hours after a barbital treatment. If the drug-free interval was increased to 72 hours, a variable reduction in sleeping time was observed after various pre-treatment schedules for 12–20 days. Although barbital sleeping times could thus measure CNS tolerance without interference by metabolism, the method has several drawbacks. It can only be used once as the test dose interferes with the tolerance mechanism and the large variability in barbital sleeping time measurements mask all but very large changes.

An increase of 35 / of barbital in the brain of rats at the end of the sleeping time has been shown to occur by REMMER *et al* (1962) after pre treatments with 200 mg/kg barbital for 5 days. This is a clear indication that a decreased sensitivity to barbital has occurred in the CNS With this method the animals can, however only be used once and the bases is still the variable sleeping times.

Various seizure thresholds have been used to measure changes in the brain excitability after barbiturate and alcohol treatment by for instance JAFFE & SHARPLESS (1965) and MCQUARRIE & FINGL (1958). With regard to barbiturates, intense pentobarbital treatments for 20 days in cats gave a reduction to 30 / of a pentylenetetrazol threshold (JAFFE & SHARPLESS 1965). These tests can be repeated with the same animal. After a treatment which induces spontaneous seizures it is, however impossible to follow any further development of increased brain excitability

With the present method it has been possible to record a gradual decrease in sensitivity to hexobarbital after prolonged barbital treatments (fig. 4) This is very probably due to a decreased sensitivity in the CNS as the time-course of the development of the increase in threshold is much slower than the time-course of increased metabolism which is fully developed after a few administrations (REMMER 1962) and there was no change in threshold in the experiments in which the sleeping times had been clearly influenced (table 3).

The slow elimination of barbital means that the thresholds recorded 24 hours after the last barbital injection probably tend to be too low as compared with the value obtained after 72 hours (fig. 2). The gradual increases in threshold dose observed during the barbital treatment (fig. 4) are probably unaffected by this source of error as maximal cumulation must already have occurred at the first determination after a week of barbital treatment.

Is the threshold increase due to a specific adaptation to a barbiturate

as such, or to the barbiturate acting as a general CNS depressant? This question of specific or non-specific cross-tolerance is an important one. Two extreme situations can be pictured The hexobarbital used to induce the silent second could substitute for barbital. The threshold increase then would be a specific measure of the decreased sensitivity to barbital the cross-tolerance would be specific. But the decreased sensitivity to hexobarbital could also be due to a general increased CNS excitability induced by the barbital pre treatment (the abstinence phase after physical dependence to barbital has been produced) Hexobarbital would then act as any other CNS depressant. In this case the threshold is an unspecific measurement which only records increased excitability in the CNS i.e. unspecific cross-tolerance That at least part of the increase in threshold seen after barbital pre-treatments need not be specific is indicated by the fact that it is also possible to induce an increased threshold to hexobarbital by long term pre-treatment with alcohol (WAHLSTRÖM 1966c).

It is interesting to note that the increase in threshold during the barbital treatment did not influence the hexobarbital sleeping times following threshold doses (fig. 6) These sleeping times are influenced by at least three factors 1) The CNS-sensitivity to hexobarbital. This also influences the return of the righting reflex which is shown by the fact that there is no positive relation between threshold dose and subsequent sleeping time in normal threshold determinations (WAHLSTRÖM 1966b) 2) The increased metabolism of hexobarbital which is induced by barbital (REMMER 1962). 3) The amount of barbital present when the threshold determination is made. The influence of the two last factors are presumably not altered after one week of barbital treatment and they could balance out, but it is impossible to evaluate each factor separately with any certainty

The increase in sleeping times after threshold doses in series XI (fig. 6 and table 6) some weeks after the end of the barbital treatment cannot be explained at present. It is evidently not a constant phenomenon as this was not seen in series XII The pre-experimental threshold doses were similar in series XI and XII but there was a marked difference in the corresponding sleeping times (table 5). After the barbital treatment the thresholds returned to the pre-experimental level in both series. The CNS sensitivity to hexobarbital determined by the threshold was thus unaltered when the sleeping times in series XI started to increase This would point to a change in metabolism although ASTON (1966) studying a similar phenomenon after pentobarbital treatment in the rat could not detect any reduced disappearance rate of pentobarbital from the blood of "post tolerant" rats. Metabolites may however have interfered with the analytical procedure used.

Summary

The changes induced by different long-term barbiturate treatments have been studied with conventional sleeping time measurements and with a threshold method. The threshold consisted of the amount of hexobarbital (enhexymalum NFN) which was needed to obtain the first burst suppression of 1 second or more in the EEG during a continuous intra venous infusion.

Pentobarbital (mebumalum NFN 30 mg/kg) administered twice daily for 5 days caused a reduction in the sleeping times obtained after a fixed dose of hexobarbital (150 mg/kg). During the treatment a reduction also occurred in the sleeping times after pentobarbital No change, however was found in the hexobarbital threshold.

Barbital (diemalum NFN 200 mg/kg) was administered once a day for 5 weeks. During this treatment the sleeping times after barbital were not altered. The hexobarbital thresholds, determined approximately 24 hours after the last barbital injection, increased gradually during the treatment. After 5 weeks of treatment this increase was approximately 40 / of the pre-experimental threshold. 72 hours after the end of the barbital treat ment the thresholds still showed an increase but after 120 hours they had almost returned to the pre-experimental value Sleeping times measured after the threshold doses were essentially unchanged during the barbital treatment although the thresholds were increased.

Acknowledgements

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Differences in Anaesthetic Properties between the Optical Isomers of 5-(2 bromoallyl)-5-isopropyl 1 methylbarbituric Acid (Enibomal NFN) in the Rat

By

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(Received M y 29 1967)

In two previous studies, differences between the anaesthetic properties of stereoisomers of N methylated barbiturates have been established (GIBSON *et al.* 1959 WAHLSTRÖM 1966a) The resolution of 5-(2-bromo-allyl)-5-isopropyl-1 methylbarbituric acid (in the following the NFN name *enibomal* is used without any further qualification for the racemate) in its optical isomers by KNABE & PHILIPSON (1966) furnished the opportunity to increase our knowledge in this field. The threshold method used previously with hexobarbital (WAHLSTRÖM 1966a) was adapted to enibomal as it belongs to the rapidly acting barbiturates. In this way a direct comparison between the isomers of hexobarbital (enhexymalum NFN) and enibomal could be made.

Methods

Male Sprague Dawley rats have been used throughout. The weight varied between 300–400 g during the experiments. The rats were kept in constant temperature room at 30 The light I the room had cycle of 12 hours of light and 12 hours of darkness and the light was put out at I P M The rats had access to water and food pellets ad libitum. All experiments were performed between 2 and 5 P M

I the threshold determinations, the barbiturates used were infused intravenously in tail vein at constant rate. The electroencephalogram was recorded on a Schwarzer electro-encephalograph during the infusion and when the first burst suppression of 1 second or more (the silent second) was noted by the observer the infusion was stopped. The dose needed to obtain the silent second was taken as the treshold. For details regarding the threshold determinations see W HLSTRÖM 1966b.

After the silent second had occurred the infusion was stopped after varying times, and the sleeping time (the time without righting reflex) was measured in constant temperature

room (30°) by means of an automatic device consisting of recording beds. All sleeping times were excluded in which more than 7.5 mg/kg of the barbiturates had been infused in addition to the dose needed for the silent second. For details regarding the sleeping time measurements see WAHLSTRÖM 1966c.

racemic Hexobarbitone sodium (evipan ®) was obtained as the commercial preparation from Bayer AG. From this a stock solution which contained 100 mg/ml was prepared with distilled water. The amount needed to give each rat a dose of 150 mg/kg was taken from this solution, and this amount was further diluted with 0.25 / sodium chloride to give a final volume of 1 ml. The infusion rate was 0.25 mg/kg/sec and the volume rate was thus 0.1 ml/min.

racemic Enibomal sodium (the sodium salt of 5-(2-bromoallyl)-5-isopropyl-1-methyl-barbituric acid, narkotal ®) was obtained as a powder from AB Astra. A stock solution which contained 100 mg/ml was prepared with 0.1 N NaOH. From this solution dose of 150 mg/kg was taken for each rat and this amount was further diluted with 0.25 % sodium chloride to give a final volume of 1 ml. Different infusion rates obtained through changes in volume rate were used (see results part 2).

The isomers of enibomal were samples of the acids obtained from Professor J. KNABE, Saarbrücken. The amount taken was that needed to give each rat a dose of 150 mg/kg calculated as the sodium salt. This was dissolved in approximately 0.6 ml of 0.5 N NaOH and diluted with distilled water to a final volume of 1 ml. The infusion rate in these experiments was 0.25 mg/kg/sec (sodium salt) and the volume rate was thus 0.1 ml/min.

All solutions were used within 1 hour of their preparation. All doses in the following are given as the sodium salt.

Results

Comparison between hexobarbital and enibomal

The rats to be used in the experiments with enibomal were first tested with hexobarbital on two occasions several days apart. The first hexobarbital threshold (the first experiment performed on these animals) was discarded (WAHLSTRÖM 1966b). The second one was used as comparison with the enibomal thresholds in the same animal. Several days after the hexobarbital thresholds, one enibomal threshold was performed with the infusion rate used in the hexobarbital threshold determinations (0.25 mg/kg/sec.) After the enibomal threshold was performed, the effect of different infusion rates on the enibomal threshold was studied (results, part 2). A new enibomal threshold determination with 0.25 mg/kg/sec. was then performed. Finally the rats were used for testing the two optical isomers of enibomal (results, part 3).

The threshold determinations with hexobarbital and enibomal were performed in the same manner. The behaviour of the rats was, however not exactly the same. During the hexobarbital infusion jerks and short convulsions sometimes occurred (WAHLSTRÖM 1966b). These were much more marked and of longer duration when enibomal was used as the threshold agent. The position of the needle in the tail vein was often

disturbed during these periods with the result that more subcutaneous infusions or totally interrupted threshold determinations were found with enibomal than with hexobarbital.

The average threshold dose of enibomal (16 rats) calculated as the mean of the two determinations with an infusion rate of 0.25 mg/kg/sec., was 72.8 ± 2.4 (s.e.m.) mg/kg. The threshold dose in the hexobarbital determination was 62.1 ± 1.9 mg/kg. Slightly more enibomal was thus needed to obtain the silent second. The variability in the enibomal treshokds also seemed to be greater the variance in the two enibomal threshold determinations were 140 7 and 149 6 (mg/kg)^2 as compared with 58.2 (mg/kg)^2 in the hexobarbital determination. There as no correlation between the dose needed with enibomal and the dose needed with hexobarbital (correlation coefficient = − 0.17 DF = 14)

2 *Studies with racemic enibomal*

Experiments with different infusion rates of enibomal were performed in order to obtain an estimate of the optimal infusion rate to get the silent second. The first infusion rate tested was 0.25 mg/kg/sec. The infusion rates were then tested in the following order 0.50 mg/kg/sec., 0.33 mg/kg/sec. 0 125 mg/kg/sec., 0 063 mg/kg/sec. and finally a second determination with 0.25 mg/kg/sec. The changes in infusion rate were all obtained by a change in volume rate. The time interval between the determinations varied between 2–12 days. It tended to be somewhat shorter between the faster infusion rates than between the slower ones.

The results are given in fig. 1 The two experiments performed with 0.25 mg/kg/sec. were averaged and taken as the basal threshold. The changes in the amount of enibomal needed to obtain the silent second were calculated as per cent of this average threshold As can be seen from fig. 1 the optimal rate was around 0 25 mg/kg/sec. The same optimal rate was found with hexobarbital (WAHLSTRÖM 1966b). The changes in the amount of enibomal needed for the different infusion rates used were also similar to those found with corresponding rates of hexobarbital.

With hexobarbital (WAHLSTRÖM 1966b) it was found that the increase in dose needed with the faster infusion rates could be fully explained by the increased amount, mainly in the veins, which had still not reached the brain when the silent second appeared The average increase in enibomal threshold dose as compared with the dose needed at 0.25 mg/kg/sec. was 2.9 mg/kg with the infusion rate of 0.33 mg/kg/sec., and 7.8 mg/kg with 0.50 mg/kg/sec. These figures are somewhat higher than the expected values (approximately 1 3 mg/kg and 3 8 mg/kg respectively) but well within the statistical error of the amounts recorded. As in the case of

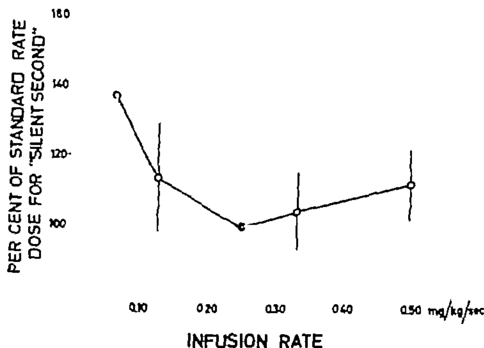


Fig. 1 The effects of different speeds of infusion on the dose needed to obtain the silent second. The concentration of enibomal was kept constant. Filled circle denotes standard rate (0.25 mg/kg/sec). Twice the s.e.m. is indicated by the bars. The number of animals was 16 for all points except for the slowest rate where it was 11. The same animals participated in all the rate experiments. Average threshold dose with standard rate was 72.8 ± 2.4 mg/kg (n = 16).

hexobarbital redistribution and metabolism of the barbiturate undoubtedly explain the steeper rise with slower rates.

After enibomal threshold doses the rats had a tendency to react to noise with a sudden jump and short convulsion. They could thus jump from the automatic recording beds and still not have regained the righting reflex. This makes the sleeping times after enibomal more unreliable than the corresponding values after hexobarbital. The sleeping times measured after enibomal thus tend to be shorter than would be the case if the righting reflex was used as a criterion.

In fig. 2 are plotted the sleeping times after different infusion rates of enibomal. There is a gradual increase with faster rates and a steeper one with slower rates. These changes are very similar to those obtained with hexobarbitone (WAHLSTRÖM 1966c). If sudden jumps and not the return of the righting reflex were the cause of the termination of all sleeping

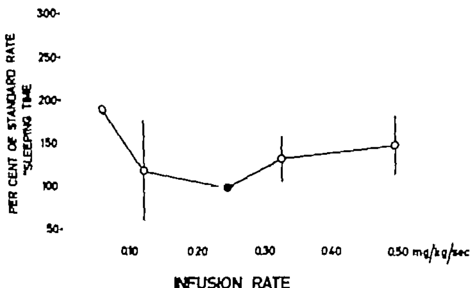


Fig . The effects of different speeds of infusion on the sleeping times obtained after threshold determinations. The concentration of enibomal was kept constant. Filled circle denotes standard rate (0.25 mg/kg/sec). The bars indicate twice the s.e.m. The umber of rats for each point starting with the slowest rate was 9 13 15 and 11 Average sleeping time with standard rate was 15.0 ± 1.4 min. (= 16). The same animals part icipated i all the rate experiments.

times after enibomal then no changes with infusion rate would have been expected. It seems therefore that noise sensitivity was not decisive in the majority of experiments.

3 *Studies with the optical isomers of enibomal*

The results with the optical isomers of enibomal are shown in table 1 The small amount of material available allowed only a few experiments. The pre-experimental average consisted of the two determinations per formed with 0.25 mg/kg/sec. of the racemate (results, part 2) (+)-enibomal was given first in rats no 2, 3 4 and 5 Two rats in which (−)-enibomal was administered first, had to be excluded as neither of the ex periments with the two isomers were technically acceptable (subcutaneous infusions) No threshold dose with (+)-enibomal was obtained in rat 2 for the same reason. The interval between the administration of the two isomers was 2–11 days.

Table 1 shows that there was a clear difference between the two

Table 1

Threshold dose and sleeping time after racemic and optically active enibomal.

Rat	Dose for silent second mg/kg			Sleeping time min.		
	Pre-exp. av	(+)	(−)	Pre-exp.	(+)	(−)
1	67 2	87 8	49 0	10.8	36.0	5 8
2	73 4	–	59.0	16.2	–	7 0
3	74 5	77.0	64.5	11.2	–	15 8
4.	61 4	87.0	61 8	7.[illegible]	7.3	4.3
5	67 9	69.0	64.5	12.8	14.2	5.5
6.	62.9	102.3	64.3	14.8	33 8	4.0
Mean	67 9	84.5	61.3	12.2	22.8	7 1
s.e.m.	2.2	5.8	2.5	1.3	7 1	1.8

Pre-exp. av = pre-experimental average with racemate.
(+) = (+)-enibomal.
(−) = (−)-enibomal.
All infusions at 0.25 mg/kg/sec.

isomers in threshold doses of enibomal needed to obtain the silent second The difference had a P value of less than 0 01 (Student s t test). This difference is, however much less marked than the one obtained with hexobarbital (WAHLSTRÖM 1966a) With (−)-hexobarbital (the least active isomer) it was possible to obtain a silent second in only one rat out of seven when the infused dose was increased by more than 90 / as compared with the racemate With the least active of the enibomal isomers, an average increase of 24 % was sufficient to give a silent second in all participating animals. With (+)-hexobarbital the threshold was reduced by approximately 30 %. The corresponding figure with enibomal was only 10 %.

During the infusion of the two antipodes of enibomal the tendency to convulsions was more marked with (−)-enibomal than with (+)-enibomal. After the infusion (−)-enibomal also seemed to make the rats more susceptible to noise. As can be seen from table 1 the sleeping times after (−)-enibomal were decreased as compared with the racemate. This is clearly different from the corresponding results with the most potent of the hexobarbital isomers (WAHLSTRÖM 1966a). The sleeping times were similar to those obtained with the racemate. The decrease in sleeping time seen after (−)-enibomal are probably due to the increased susceptibility to noise. After (+)-enibomal the sleeping times were increased

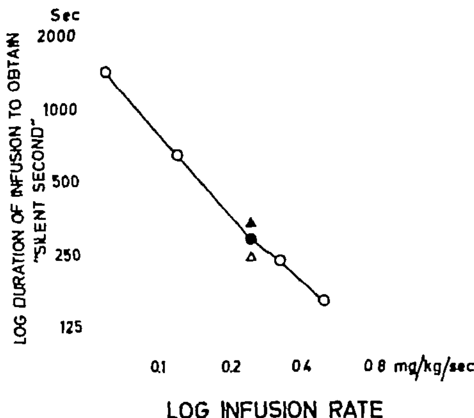


Fig. 3 The influence of infusion rate on the time needed to obtain the silent second. Circles denote the experiment in fig. 1 Open triangle denotes the experiments with (−)-enibomal, and filled triangle the experiments with (+)-enibomal. For further explanation see text.

(table 1). The less potent of the hexobarbital isomers also gave very long sleeping times. It is not known whether the increase seen in the present experiments with enibomal is due to an unknown factor similar to the one in the hexobarbital experiments or simply to a decreased tendency to end the sleeping time by a sudden jump These two alternatives are, however not mutually exclusive.

Infusion of enibomal with different rates increases the threshold dose needed to obtain the silent second, if the rate is changed from the optimal one around 0.25 mg/kg/sec. (results, part 2) In the experiments with the isomers of enibomal the "anaesthetic activity" is infused more slowly with (+)-enibomal (18 / of the threshold dose needed was infused per minute) and more quickly with (−)-enibomal (25% of the threshold dose

Table I

Threshold dose and sleeping time after racemic and optically active enibomal.

Rat	Dose for silent second mg/kg			Sleeping time min.		
	Pre-exp av	(+)	(−)	Pre-exp v	(+)	(−)
1	67.2	87.8	49.0	10.8	36.0	5.8
2.	73.4		59.0	16.2	–	7.0
3	74.5	77.0	68.5	11.2		15.8
4	61.4	87.0	61.8	7.2	7.3	4.3
5	67.9	69.0	64.5	12.8	14.2	5.5
6.	62.9	10[illegible]3	64.3	14.8	33.8	4.0
Mean	67.9	84.5	61.3	1[illegible]2	22.8	7.1
s.e.m.	[illegible]2	5.8	2.5	1.3	7.1	1.8

Pre-exp. av = pre-experimental average with racemate
(+) = (+)-enibomal
(−) = (−)-enibomal
All infusions at 0.25 mg/kg/sec.

isomers in threshold doses of enibomal needed to obtain the silent second. The difference had a P value of less than 0.01 (Student's t test). This difference is, however, much less marked than the one obtained with hexobarbital (WAHLSTRÖM 1966a). With (−)-hexobarbital (the least active isomer) it was possible to obtain a silent second in only one rat out of seven when the infused dose was increased by more than 90% as compared with the racemate. With the least active of the enibomal isomers an average increase of 24% was sufficient to give a silent second in all participating animals. With (+)-hexobarbital the threshold was reduced by approximately 30%. The corresponding figure with enibomal was only 10%.

During the infusion of the two antipodes of enibomal the tendency to convulsions was more marked with (−)-enibomal than with (+)-enibomal. After the infusion (−)-enibomal also seemed to make the rats more susceptible to noise. As can be seen from table I the sleeping times after (−)-enibomal were decreased as compared with the racemate. This is clearly different from the corresponding results with the most potent of the hexobarbital isomers (WAHLSTRÖM 1966a). The sleeping times were similar to those obtained with the racemate. The decrease in sleeping time seen after (−)-enibomal are probably due to the increased susceptibility to noise. After (+)-enibomal the sleeping times were increased

KAMP 1965) In the systems used (ether dimethylformamide and water or ether petroleum ether dimethylformamide and water) enibomal had distribution coefficients close to hexobarbital and larger than propallylonal. A fast N-demethylation of (+)-enibomal could thus change it into a substance which due to a long latency would have a high threshold Fast infusion rates would decrease the opportunity for the N-demethylation of the (+)-enibomal in the racemate. As a result the potency of the racemate would increase and the thresholds decrease with faster rates. This was, however not the case (results, part 2) This does not exclude a fast N-demethylation but makes it unlikely as an explanation for the differences in potency between the isomers of enibomal.

Different protein binding properties could also explain the difference in potency between the isomers of enibomal. Protein binding, however does not seem to be the crucial factor in limiting the amount which penetrates into the brain since thiopental, which is a very rapidly acting barbiturate was found by GOLDBAUM & SMITH (1954) to be bound to albumin to a greater extent than any of the other barbiturates used for comparison.

The cause of the difference between the isomers of enibomal is thus probably connected with the CNS either at the site of action or as an active stereo-selective process connected with the penetration into the brain. The fairly good correlation which exists between penetration into the brain and lipid solubility among the barbiturates (BUSH 1963) would make an active penetration process the more unlikely of the two alternatives.

(+)-enibomal which was the least potent of the antipodes with regard to threshold dose, also seemed to give less jerks and convulsions during the infusion. It is thus unlikely that the small potency of (+)-enibomal is due to a concomitant opposite effect, CNS excitation.

All barbiturates with an N-methyl group have an asymmetric carbon atom in the 5 position of the ring, if the two substituents there are different. An earlier study with 1-methyl 5-n-butyl 5-ethyl-barbituric acid showed no convincing difference in the median anaesthetic dose (KLEIDERER & SHONLE 1934). Differences in anaesthetic properties have however been found between the enantiomorphs of 1 methyl-5-(1-methyl-2 pentynyl)-5-allyl-barbituric acid (GIBSON *et al* 1959) a substance which also has an asymmetric carbon atom in one of the substituents, and between the isomers of hexobarbital (WAHLSTRÖM 1966a, RUMMEL *et al* 1967)

The isomers of hexobarbital have been tested by the present method The only difference between these two sets of experiments was the time in the 24-hour light-darkness rhythm at which the experiments were performed The present experiments were done during the first hours of darkness. The relative potencies of the isomers as compared with the

racemate can hardly be influenced by this difference. There was, further more, only a slight difference between the hexobarbital threshold determined during the first hours of darkness in this experiment (62.1 mg/kg) as compared with the corresponding thresholds determined during the light (66.8 mg/kg n = 110) in a rhythm of 12 hours of light and 12 hours of darkness (WAHLSTRÖM 1966b)

The results with enibomal were similar to those obtained with hexobarbital but the differences between the isomers were less marked (results, part 3) The difference on the whole between the two substituents in the 5 position is less marked in enibomal than in hexobarbital which would tend to give the hexobarbital more marked steric properties on biological receptors. This could explain the smaller difference between the isomers of enibomal as compared with hexobarbital. Further discussion on this point will have to wait until the absolute configuration of the isomers of hexobarbital and enibomal have been determined. Whether (+)-hexobarbital and (−)-enibomal, the two most potent isomers are sterically related or not is an important question.

Summary

The racemate and the two antipodes of 5-(2-bromoallyl)-5-isopropyl-1 methyl-barbituric acid (enibomal NFN) have been tested by means of an EEG threshold method The threshold consisted of the amount of enibomal which was needed to obtain the first burst suppression of 1 second or more in the EEG during a continuous intravenous infusion.

During the threshold determination *racemic* enibomal was found to cause more jerks and short convulsions than *racemic* hexobarbital. Different infusion rates were studied and an optimal rate around 0.25 mg/kg/sec. was found. With faster and slower rates the thresholds increased The sleeping times after the threshold doses of racemic enibomal were influenced by the infusion rates in the same manner as the thresholds.

There was a *difference in the thresholds* obtained with the two isomers of enibomal. On an average, 61 mg/kg of (−)-enibomal was needed as compared with 85 mg/kg of (+)-enibomal The isomers seemed to be directly additive. The sleeping times after threshold doses increased in the following order (−)-enibomal, racemate and (+)-enibomal. The tendency to convulsions was more marked with (−)-enibomal than with (+)-enibomal.

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Differences in Tolerance to Hexobarbital (*Enhexymalum NFN*) after Barbital (*Diemalum NFN*) Pre-treatment during Activity or Rest

By

Göran Wahlström

(Received M y 29 1967)

Prolonged barbital (diemalum NFN) treatment causes a decreased sensitivity to hexobarbital (enhexymalum NFN) as measured by an EEG threshold (WAHLSTRÖM 1968). SEEVERS & DENEAU (1963) suggested that such a tolerance to barbiturates could be explained by *either of two* different mechanisms. One was called "learning" and the other "cellular tolerance" The present experiments were devised to differentiate between *these two alternatives. As the learning" situation should be more marked* during the activity of the animals a larger tolerance would be expected if the maximal effect of the inducing barbiturate occurred during activity Consequently tolerance treatment was performed at two different phases of the activity cycle. There was a greater tolerance if treatment was given during activity than during rest.

Methods

Male Sprague-Dawley rats (Anticimex) were used The a erage pre-experiment weights were between 115–339 g and the verage weights, before the last threshold determination, were between 391–414 g in the different groups used. The rats were kept in rooms t a constant temperature of 30 The light cycle consisted of 12 hours of light and 12 hours of darkness. In the series where the rats were kept in the same room (SR-series) light was *turned on at 8.00 a.m. In the series where the rats were kept in d fferent rooms* (DR-series) the light was turned on at 8.00 a.m. in the room where the animals treated during light were kept and at 1.00 a m. in the room where the animals treated during darkness were kept. The rats had access t water and food pellets ad libitum. All treatments and experiments were performed in the first 5 hours of light in the animals treated during light (L-treated) and in the first 5 hours of da kness in the animals treated during darkness (D-treated).

Threshold determinations

Hexobarbital was infused into tail vein at a constant rate. *Racemic* Hexobarbital sodium (evipan ®) was obtained as powder in the commercial preparation from Bayer AG. A stock solution (to be used within an hour after preparation) with a concentration of 100 mg/ml of the salt was prepared with distilled water. From this solution, the amount needed to give each rat a dose f 150 mg/kg was taken. It was further diluted with 0.25 % sodium chloride up to a final volume of 1 ml. The rate of the hexobarbital infusio was 0.25 mg/kg/sec. and the volume rate thus 0.1 ml/min. During the infusion the electroencephalogram was recorded Schwarzer electroencephalograph. The first burst suppression of 1 second or more was taken as the end point (the silent second), and the dose of hexobarbital needed to btain this silent second was taken as the threshold. Details about this method have been given previously (WAHLSTRÖM 1966a).

Sleeping time determinations

When the silent second had occurred the infusion was stopped. The amount of hexobarbital infused after the silent second depended on whether the first silent second in the EEG-curve was observed during the experiment not. The ensuing sleeping time (the time without righting reflex) was measured in one of the rat rooms with an automatic device. This consists of recording beds, which the animals leave when the righting reflex returns. All sleeping times were discarded in which more than 7.5 mg/kg of hex barbital had been infused after the silent second. For details about the sleeping times, see WAHLSTRÖM 1966b.

Sleeping times were also recorded fter administration of fixed dose of barbital sodium (200 mg/kg). Barbital sodium was always given as an intraperitoneal jection in the lower left part of the abdomen. The solution which contained 40 mg/ml was prepared immediately before use with distilled water. All control groups received 0.9 % sodium chloride instead of barbital.

Activity measurements

The sound in the rat room was recorded with tape recorder for 30 seconds with intervals of 20 mi utes. At the beginning and end f each magnetic tape used, the noise from ordinary buzzer (L M Ericsson KLG 1257 24 V) was recorded for t least mi ute, and used for calibration in the integration process. The tape recorded signals were rectified and smoothed with RLS-filter (I response t a tone burst on the input the output h d 10–90 % rise-time of 25 msec. and 10–90 % falltime of 900 msec.). The output signal was integrated during 30 seconds (the total time f each record) As an integrator volt to-frequency converter was used and the output frequency was counted by means f an electro-mecha ical counter. This device has proved t be roughly linear in the conversion of electric nput to frequency output (EKSTEDT & STÅLBERG, unpublished). The calibration noise was always set to give 200 impulses/minute. Under these conditions 1 2 impulses were obtained during the periods f 30 seconds without any recorded rat noise, interval which always separated the actual records.

C-barbital experiments

10μc/kg ^{14}C-barbital (Tracerlab) was injected intraperitoneally together with unlabelled barbital. The dose was 200 mg/kg and the concentration 40 mg/ml. The i jection was done 1 hour fter the light was turned on in the L-treated group and 1 hour after the light was turned off the D-treated group. The animals were killed after pre-determined times and the blood vessels of the brain emptied by a fast infusion of commercial Ringer (salidex ®),

AB Pharmacia) in the heart. The brains were then taken out and homogenized. Approximately 100 mg of the homogenate were digested in 1 ml of hyamine ® (Packard Co)/ toluene (1 1 v/) for approximately 7 hours at 70 10 ml of scintillation fluid (500 ml toluene, 500 ml dioxane, 300 ml methanol, 104 g naphtalene. 6.5 g PPO (Packard Co.) and 0.135 g dimethyl-POPOP (Packard Co.) were added. Duplicates from each animal were prepared and the average was used. Counting efficiency was approximately 65 calculated by the addition of an internal standard. At least 10000 counts/sample were counted.

All doses are given as the sodium salts.

Results

A Activity under the environmental conditions used

To obtain a crude estimate of the activity of the rats with the light and temperature conditions used the noise in the rat room was recorded as described. Records were only obtained during Saturdays and Sundays when no experiments were performed. The rats recorded belonged to series SR (the rats kept in the same room) and all data used were gathered before the barbital treatment. The results are given in fig. 1 All recording

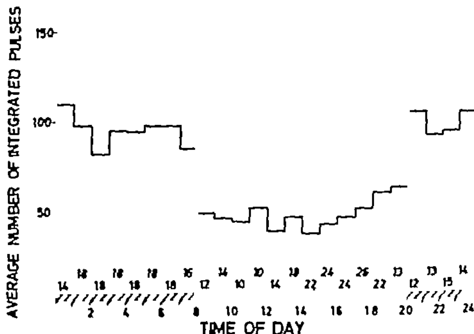


Fig. 1 Average activity in the rat room measured as integrated noise level during a 24-hour cycle Records of duration of 30 seconds were obtained every 20 minutes. The number of days on which recordings were done was 6–9 The number of records on which the average is calculated is shown in the lower part f the figure. The stippled bar along the axis indicates light off unfilled bar indicates light on. For further explanation see Methods.

Table 1

Pre-experimental values of dose for silent second in mg/kg.

Treatment		Same Room (SR)			Different Rooms (DR)			Total		
		Mean	s.e.m.	n	Mean	s.e.m.	n	Mean	s.e.m.	n
L-treated	Barbital	66.2	2.2	11	63.1	1.8	9	64.8	1.4	20
	NaCl	70.7	1.0	18	63.1	2.1	9	68.2	1.2	27
	Total.							66.7	0.9	47
D-treated	Barbital	64.6	1.5	14	60.8	1.1	12	62.8	1.0	26
	NaCl	65.1	1.5	12	61.2	0.9	15	62.9	0.9	27
	Total							62.9	0.7	53

L-treated = treated during light.
D-treated = treated during darkness.

periods which occurred during the indicated hour have been averaged Fig. 1 shows that the noise level measured as integrated pulses per re cording period, were approximately twice as high during almost total or total darkness as during light. The slight increase in noise level before the onset of darkness could indicate that the rats expected the darkness but the recording method is too crude for any definite conclusions. The shift between light and darkness was abrupt. Thus the noise level in the rat room as expected showed that with the present environmental conditions the main activity of the rats occurred during the dark period

B Increases in hexobarbital thresholds induced by barbital treatments during light or darkness

Before the start of the barbital treatment at least four threshold deter minations were performed in all animals. The first determination was discarded as it tends to give too high values of the thresholds dose (WAHLSTRÖM 1966a). The following three determinations were used to calculate a pre-experimental value of the threshold dose. If any of these had to be discarded due to technical reasons (mainly subcutaneous in- fusions) more determinations were performed until there were three determinations which were acceptable. In series SR, however there were only two acceptable pre-experimental determinations in 6 rats all belong ing to the two control groups. All pre-experimental threshold determina- tions were performed at the times later used during the experiment namely

Table 2

Barbital sleepi g times in minutes after 200 mg/kg i p.

Series	Treated during	Barbital treatment number								
		1						6		
		Mean	n	s m.	Mean	n	s.e m	Mean		s.e.m.
SR	Light	13	9	41	274	9	56	72	11	33
DR	Light	37	9	7						
Both	Total for light	225	18	4						
SR	Darkness	50	8	44	18	12	37	707	10	70
DR	Darkness	167	1	8						
Both	Total for darkness	200	70	5						

None of the differences were significant ($P < 0.05$ Student s t test)

Rats with sleeping times shorter than 10 min. were excluded because of the slow loss of the righting reflex after barbital.

the first five hours of darkness in the groups treated during darkness (D-treated) and the first five hours of light in the groups treated during light (L-treated) The time interval between the threshold determinations was usually about a week (range 2–20 days) The rats were allocated at random to the different treatments after the first threshold determination was performed

The average *pre-experimental* value of threshold doses is given in table 1 The mean obtained for each rat from 2–3 determinations was used as the statistical unit. Table 1 shows that there is a difference in threshold dose between the D-treated and the L-treated animals ($P < 0.001$) when the total material was used This difference is mainly due to a high value in the control rats in the L treated group of series SR. The average difference between the animals treated later with barbital during light or darkness was much smaller (2.0 mg/kg) and was not significant statistically In the D-treated groups there was no difference between barbital and saline treated animals. There was a difference in the corresponding L-treated groups due to the control in series SR. A slightly lower threshold was thus obtained in the animals where the threshold determinations were performed during the dark period In the barbital treated groups this difference did not amount to more than 3%.

Barbital in a dose of 200 mg/kg or the corresponding volume of 0.9 / NaCl was administered approximately 1 hour after the start of light or darkness. A threshold determination was performed every week On these

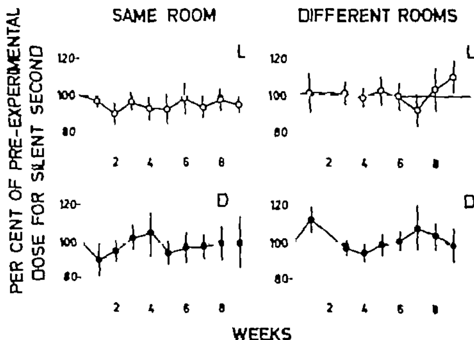


Fig. 2. Thresholds doses of hexobarbital in the control groups during (filled bar in the lower part of the figure) and after treatment with saline. The bars at each point indicates twice the standard error of the mean. The pre-experimental values for threshold dose are given in table 1. The number of animals in each point was in series SR (same room) L-treated 14–17 D-treated 7–11 and in series DR (different rooms) L-treated: 8–9 and D-treated 9–13. D = treated during darkness (D-treated). L = treated during light (L treated)

days barbital or saline was administered after the threshold determinations had been performed, if the treatment was to continue.

In table 2 the sleeping times induced by barbital injections during the first part of the treatment is shown. The sleeping times were very variable which is due to the slow loss and return of the righting reflex after barbital. None of the differences seen between the various groups were statistically significant. With regard to sleeping time after barbital treatment, there was thus no demonstrable difference between the groups treated during light or darkness. The sleeping times were not followed during the treatment but earlier experiments with the same treatment during light have shown that there was no reduction in sleeping times (Wahlström 1968).

Several rats died during the barbital treatment. In series SR the numbers were 11 and 16 in the L- and D-treated groups respectively. In series

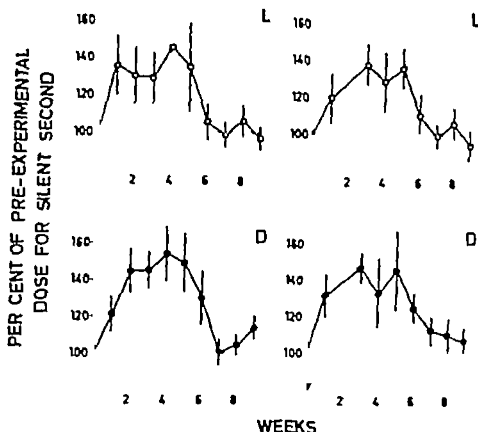


Fig. 1. Threshold doses of hexobarbital during (filled bar in the lower part of the figure) and after treatment with barbital 200 mg/kg i.p. The bars at each point indicate twice the standard error of the mean. The pre-experimental values for threshold doses are given in table 1 The number of animals were in series SR (same room) L-treated 8–10, D-treated 9–13 and in series DR (different rooms) L treated 6–9 D-treated 9–11 D = treated during darkness (D-treated). L = treated during light (L-treated).

DR the corresponding numbers were 14 and 15 These animals did not differ from the animals which survived the treatment with regard to the pre-experimental threshold dose or first barbital-induced sleeping time. There was thus no difference in this respect between light and dark treated animals. Among the controls 5 died in series SR and 3 in series DR. Autopsy were performed on all but 16 rats where the postmortem changes were very extensive (due to the temperature in the rooms). Macroscopic examination was performed and the only positive findings were patchy

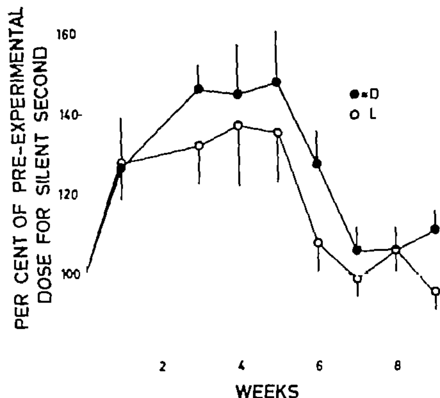


Fig. 4 Difference in hex barbital threshold changes induced by barbital treatment during light or darkness. Series SR and DR in fig. 3 have been pooled. Filled bar in the lower part of the figure indicates barbital treatment. The bars at each point plotted only in one direction indicates twice the standard error of the mean. The average pre-experimental values are given in table 1 The number f animals in each point was in the D-treated (D) = 19–23 and in the L-treated (L) = 15–18.

white infiltrates in the lungs in 8 rats, liver changes in one rat and damage due to the intraperitoneal injections in 3 rats.

The results of the threshold determination in the control groups are shown in fig. 2. The average difference from the pre-experimental value was more than 10 / on only two test occasions. No general trend could be seen except in the animals treated during light in series SR where all tests showed lower values than the pre-experimental one. As mentioned earlier this value was remarkably high (table 1).

The results of the threshold determination with hexobarbital in the different barbital treated groups are shown in fig. 3 It is evident that all groups responded to the barbital treatment with an increase in threshold

dose. After 5 weeks of treatment the increase was 30–50%. There was no great difference between the L-treated groups in the two series nor between the D-treated groups. If the results with the different groups are studied closely some consistent differences between L and D-treated groups are obtained These are better demonstrated in fig. 4 where all animals treated during darkness, and all animals treated during light also have been pooled

Fig. 4 shows that there were differences in the amount of hexobarbital needed to obtain the silent second in the animals treated with barbital during light as compared with animals treated during darkness. After more than one week of barbital treatment more hexobarbital was needed in the D-treated group on every test occasion during the treatment. This difference was only satistically significant ($P < 0.02$) after three weeks of treatment. It was however so consistent that it can be stated that treatment with daily injections of barbital 200 mg/kg for more than one week will give a greater increase in hexobarbital threshold if the barbital injections are performed during activity (darkness) as compared with similar treatment during rest (light) This increased threshold was not restricted to the threshold determinations performed approximately 24 hours after the last barbital injection. One week after the end of the barbital treatment the D-treated animals still needed more hexobarbital. This difference had a P value of less than 0 01 The increased hexobarbital threshold was thus present for a much longer time when the barbital treatment was performed during activity (darkness)

The sleeping times after threshold doses of hexobarbital were very variable and no consistent changes were observed.

C Experiments with ¹⁴C-barbital injected during light or darkness

The disappearance rates of barbital from the brain after a single intraperitoneal injection performed either at the beginning of the light period or at the beginning of the dark period have been tested with ^{14}C barbital. Fig. 5 shows that there was a difference in the disappearance rate of ^{14}C barbital between the two different times of administration. The disappearance in both sets of experiments seemed to be linear with time in the interval tested The regression coefficient was -0.585 ± 0.040 in the experiments where barbital was administered early in the light period (L-treated) and -0.401 ± 0.028 in the corresponding D-treated experiments. The difference between these two regression coefficients had a P value of less than 0 01 (Each animal is one statistical unit).

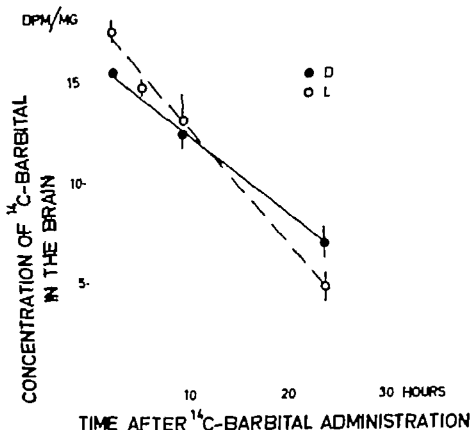


Fig. 5 Amount of ^{14}C-barbital in the brains at various times after administration. 10 µc/kg ^{14}C-barbital was given either 1 hour after light was turned off (D) or turned on (L). The total dose of barbital was 200 mg/kg. The number of animals in each point in the L-experiment was 4, 4, 8 and 4 and in the D-experiments 3, 8 and 4 starting with the shortest time interval. The bars indicate twice the standard error of the mean.

Discussion

The increase in hexobarbital threshold induced by the barbital treatment could either be caused by a change in metabolism (REMMER 1962 CONNEY & BURNS 1962) or by a change in CNS sensitivity In an earlier paper (WAHLSTRÖM 1968) it was shown that the increased threshold induced by barbital treatment during light was probably due to a change in CNS sensitivity as the time course of increase in hexobarbital threshold after barbital is different from the time course of induced faster metabolism and a barbiturate treatment which induced clear metabolic tolerance did not influence the threshold

The differences found between the threshold increases after barbital treatment during light or darkness (results, part B) can be explained in two different ways. One possibility is that barbital administered during darkness gives higher concentrations of the drug in the CNS and thus causes a more marked and longlasting increase in hexobarbital threshold If such were the case the present findings could perhaps be explained by cellular tolerance.

The other possibility is that the concentrations of barbital obtained at the two different times of administration are roughly the same but the propensity of the animals to react with increased hexobarbital thresholds is greater when they are sedated during their activity phase (equivalent to darkness, results, par A) Thus the animals would in some way try to counteract the effects of the barbital or to quote SEEVERS & DENEAU (1963) exhibit a "compensatory adaptation by secondary neuronal circuits in the CNS which counteracts the depression of barbiturates on the cells of the primary site of action (this latter mechanism could be termed "learning")"

The increase in threshold is of course dependent on the concentration of barbital to which the brain has been exposed during the development of tolerance Treatment for five weeks with 100 mg/kg barbital daily increased the threshold by approximately 20 / (WAHLSTRÖM unpublished) as compared with a corresponding increase of 30–50 / following 200 mg/kg in the present experiments.

The ^{14}C-barbital experiments were performed to see whether brain concentration was the important factor in the difference between the dark and light treatment. As barbital is metabolized only to a small extent in the rat (GOLDSCHMIDT & WEHR 1957 EBERT *et al* 1964 BURNS *et al* 1957) the radioactivity counted must with reasonable certainty be unchanged barbital These experiments gave no clear cut results because the disappearance lines intersected (part C) There was a faster disappearance rate when barbital was administered during light. The concentration in the brain was, however not consistently higher after administration during darkness. During the first 10–12 hours after administration the concentration on the contrary was smaller as compared with administration during light. Less barbital must have been available for penetration into the brain after intraperitoneal administration during activity but the subsequent elimination was slower Thus ^{14}C barbital experiments did not show such differences in brain concentrations between the two times of administration as to explain the difference in hexobarbital threshold. As we do not know the significance of the slightly higher concentration of barbital remaining in the brain late after administration during darkness,

a difference in brain concentration can not be completely ruled out as an explanation of the present result. Thus it is probable, but not definitely proved that development of tolerance to barbital contains at least an element of "learning" in the sense described by SIEVERS & DENEAU (1963).

Summary

The changes induced in hexobarbital threshold by barbital treatment at different times in the activity-rest cycle have been studied. The threshold consisted of the amount of hexobarbital which was needed to obtain the first burst suppression of 1 second or more in the EEG during a continuous intravenous infusion. That activity occurred during darkness in the 12 12 darkness-light cycle used was established by means of records of the noise in the rat room.

Barbital (200 mg/kg) was given daily for 5 weeks either early in the light period (L-treated) or early in the dark period (D-treated) Hexobarbital threshold determinations every week during the treatment showed that after 2 weeks of barbital treatment there was a consistent difference between L and D-treated animals. The D-treated needed approximately 10 % more hexobarbital. One week after the end of the barbital treatment, the thresholds of the L-treated animals were of approximately pre-experimental magnitude. In the D-treated animals however there was still an increase in threshold of approximately 25 %.

^{14}C-barbital (after a single administration of 10 μc/kg) disappeared at a slower rate from the brains of the D-treated, as compared with the L treated animals. During the first 10–12 hours after administration, however the barbital concentration was lower in the D-treated. It is thus doubtful whether concentration differences could explain the differences in threshold.

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This study was supported by the Swedish Medical research Council as project number B67 14X-9 and the Tri-Centennial Fund of the Bank of Sweden.

The differences found between the threshold increases after barbital treatment during light or darkness (results, part B) can be explained in two different ways. One possibility is that barbital administered during darkness gives higher concentrations of the drug in the CNS and thus causes a more marked and longlasting increase in hexobarbital threshold. If such were the case the present findings could perhaps be explained by cellular tolerance

The other possibility is that the concentrations of barbital obtained at the two different times of administration are roughly the same but the propensity of the animals to react with increased hexobarbital thresholds is greater when they are sedated during their activity phase (equivalent to darkness, results, par A) Thus the animals would in some way try to counteract the effects of the barbital or to quote SIEVERS & DENEAU (1963) exhibit a compensatory adaptation by secondary neuronal circuits in the CNS which counteracts the depression of barbiturates on the cells of the primary site of action (this latter mechanism could be termed "learning")"

The increase in threshold is of course dependent on the concentration of barbital to which the brain has been exposed during the development of tolerance. Treatment for five weeks with 100 mg/kg barbital daily increased the threshold by approximately 20 / (WAHLSTRÖM unpublished) as compared with a corresponding increase of 30-50 / following 200 mg/kg in the present experiments.

The [14]C barbital experiments were performed to see whether brain concentration was the important factor in the difference between the dark and light treatment As barbital is metabolized only to a small extent in the rat (GOLDSCHMIDT & WEIR 1957 EBERT *et al* 1964 BURNS *et al.* 1957) the radioactivity counted must with reasonable certainty be unchanged barbital These experiments gave no clear cut results because the disappearance lines intersected (part C) There was a faster disappearance rate when barbital was administered during light. The concentration in *the brain was, however not consistently higher after administration* during darkness. During the first 10–12 hours after administration the concentration on the contrary was smaller as compared with administration during light. Less barbital must have been available for penetration into the brain after intraperitoneal administration during activity but the subsequent elimination was slower Thus [14]C barbital experiments did not show such differences in brain concentrations between the two times of administration as to explain the difference in hexobarbital threshold. As we do not know the significance of the slightly higher concentration of barbital remaining in the brain late after administration during darkness,

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Impairment of Renal Concentrating Capacity in Albino Rats Induced by Phenacetin and Acetylsalicylic Acid

By

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(Received April 28, 1967)

During the last few years there has been an increasing interest in nephrotoxic substances. Several animal experiments suggest a nephrotoxic effect from phenacetin and N-acetyl-p-aminophenol (NAPA) (THÖLEN *et al.* 1956 MIESCHER *et al* 1958 EISALO & TALANTI 1961 ANGERVALL *et al.* 1962a, b & c & 1964 SCHOURUP *et al* 1963 ABRAHAMS *et al.* 1963 & 1964 PRZYBYL *et al* 1964 CLAUSEN 1962 & 1964 FORDHAM *et al* 1965). Some experiments also suggest a nephrotoxic effect with acetylsalicylic acid (STUDER *et al* 1958 CLAUSEN 1962 & 1964).

In a previous study (ANGERVALL *et al* 1964), we found that the renal concentrating capacity was significantly lower in rats given phenacetin or NAPA, in daily doses of approximately 0 4 g/kg body weight for 41 weeks than in the control rats. Rats receiving 0.25 g acetylsalicylic acid/kg body weight for the same number of weeks, exhibited an insignificant decrease in concentrating capacity The reason for choosing a lower dose of acetylsalicylic acid was because of a previous experiment which demonstrated general toxic effects at higher doses. After the concentrating test, the rats were inoculated with E. coli and thus a possible parenchymal renal lesion directly related to the drug intake could not be evaluated

We therefore found it desirable to perform further rat experiments with long-term feeding with phenacetin and acetylsalicylic acid. Our aims were 1) to measure the renal concentrating capacity in each rat repeatedly during the experimental period 2) to find out whether an induced decrease in the renal concentrating capacity would be normalized after discontinuing the drugs 3) to examine the kidney morphology

The results of the renal function studies are described here.

Material and Methods

Forty-eight female rats of a Sprague Dawley strain supplied by Anticimex AB, Stockholm were used. The initial weight of the rats was about 190 g. The rats were put on a powdered standard food, the composition of which was the same as described in an earlier study (ANGERVALL *et al* 1962b), except that "Ewomin purum" was reduced to 0.1 kg. After two weeks on this diet, the renal concentrating capacity was estimated in all rats. The rat were then divided into th f llowing groups

Group I: Controls, i.e. rats recei ing the powdered food with no drug added (16 rats).
Group II Rats receiving the powdered food to which phenacetin was added in an amount of 5.35 g/kg food (16 rat)
Group III Rats receiving the powdered food, to which acetylsalicylic acid was added, in an amount of .70 g/kg food (16 rats).

The rats had free access to food and tap water After 40 weeks on this regimen 11 rats in group I 12 in group II and 13 in group III were killed and autopsied. In the remaining rats the test was continued for 3 more weeks. The drugs were then discontinued. After 3 *additional weeks with co trol food f r all three groups, the remaining 12 rats were killed* and autopsied.

Throughout the experimental period, the food intake per week was estimated for each group, and each rat weighed once a week. The renal concentrating capacity was tested in the same way as in our previous study (ANGERVALL *et al* 1964). Water was withheld for 18 hours, from 9 p.m. until 3 p.m the followi g day Throughout this test the rats were kept in individual cages with a fine-meshed bottom to facilitate the removal of the faeces. Below the grid there was funnel discharging the urine into a flask with a narrow orifice. The osmolality was determined in urine collected from 9 a.m. to 3 p.m., i.e the last 6 hours of w ter withdrawal. The osmolality was calculated from the freezing point depression, measured with thermistor and a resistance bridge. This procedure was repeated at intervals as shown in tables I and .

Results

The curves in fig. 1 and 2 cover the period during which the rats in groups II and III were given drugs.

Dietary intake curves are given in fig. 1 The food intake was greatest for the phenacetin group but there was no difference between the acetylsalicylic acid group and the control group After discontinuation of the drugs the food intake was similar for the three groups (with reduced number of rats).

The approximate drug intake as calculated from the average weekly food intake and the mean body weight was 0 45 g of phenacetin for group II and 0.25 g of acetylsalicylic acid for group III per kg body weight per day

Mean body weight curves are shown in fig. 2. There was no definite difference in weight gain between the rats in the three groups. Although the phenacetin group *is represented* by the lowest of the three parallel curves, the mean body weight in this group was only 4 g (1 4 %) lower at the end of the drug feeding period, as compared with the controls.

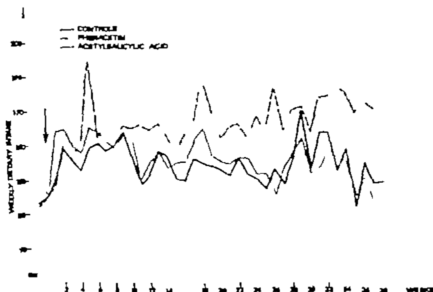


Fig. 1 Dietary intake in rats during prolonged administration of drugs. Arrow indicates start of drug feeding phenacetin 5.35 g/kg food or acetylsalicylic acid 2.70 g/kg food. Controls received no drugs.

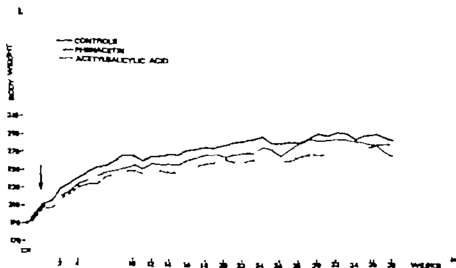


Fig. 2. Mean body weight in rats during prolonged administration of drugs. Arrow indicates start of drug feeding: phenacetin 5.35 g/kg food or acetylsalicylic acid 2.70 g/kg food. C ntrols received no drugs.

Table 1

The mean renal concentrating capacity in the various test groups during the period of drug consumption.

Group	No. of rats	Maximal urine osmolality mOsm/kg H_2O (mean and standard error of mean)							
		Before drug is added	Period of drug added (weeks)						
			1	9	13	18	6	34	38
I	16	2625 ± 53	2676 ± 61	2796 ± 51	2769 ± 64	2545 ± 153	2756 ± 92	[illegible]648 ± 89	254 ± 101
II	16	2692 ± 83	2739 ± 76	2578 ± 128	2571 ± 85	2544 ± 99	660 ± 107	[illegible]81 ± 103	2264 ± 58
III	16	2532 ± 81	2577 ± 84	2384 ± 70	2440 ± 74	2440 ± 70	2346 ± 63	[illegible]79 ± 76	113 ± 77

I Controls – II Phenacetin III Acetylsalicylic acid

Table 2

Group	No. of rats	Maximale urine osmolality mOsm/kg H_2O (mean and standard error of mean)						
		Period of drug consumption (weeks)		Various times after withdrawal of the drugs (weeks)				
		38	41	1	3	7	12	18
I	4	[illegible]592 ± 220	2872 ± 76	2732 ± 152	2942 ± 101	3133 ± 408	2592 ± 156	2750 ± 153
II	5	2398 ± 81	2264 ± 125	2466 ± 201	2901 ± 231	442 ± 236	[illegible]354 ± 184	2786 ± 193
III	3	2027 ± 102	2170 ± 112	2624 ± 116	2396 ± 11	2564 ± 104	2249 ± 88	2478 ± 72

I Controls – II Phenacetin – III Acetylsalicylic acid.

The renal concentrating capacity measured as urine osmolality is shown in table 1.

After 38 weeks, there was a significantly lowered osmolality in both the phenacetin group (2264 mOsm/kg H_2O, $P < 0.001$) and the acetylsalicylic acid group (2113 mOsm/kg H_2O, $P < 0.001$) as compared with the mean osmolality in these groups before the drugs were added (2692 and 2532 mOsm respectively) as well as compared with the mean osmolality in the control group after 38 weeks (2542 mOsm, $P < 0.001$). The mean osmolality of the latter group did not change significantly during the experimental period.

Table 2 gives the mean osmolality for the remaining rats in the three groups after 38 and 41 weeks of drug administration and at various intervals after discontinuation of the drugs. After 41 weeks there was a significantly lower osmolality in both the phenacetin group (2264 mOsm) and the acetylsalicylic acid group (2170 mOsm) as compared with the control group (2872 mOsm), $P < 0.05$ and $P < 0.001$ respectively. Eighteen weeks after discontinuation of the drugs, no significant difference in mean osmolality could be demonstrated between the three groups.

Discussion

The aim of the present study was to follow the renal concentrating capacity in rats during long-term feeding with phenacetin and acetylsalicylic acid. The doses given were estimated to be the highest possible doses which would not produce general toxic effects. In a previous experiment (ANGERVALL *et al.* 1962b), it was found that acetylsalicylic acid tended to cause general toxic effects at lower doses than phenacetin. Rats receiving 0.5 g of acetylsalicylic acid daily per kg body weight developed hyperphagia, weight loss, intestinal bleeding and died prematurely. The course of the dietary intake and the weight curves in the present study suggest that such toxic effects have been avoided. There were no spontaneous deaths.

The urine osmolality in the drug fed rats was not significantly lowered until the 34th week. This result is in accordance with clinical experience that long term consumption is necessary to cause renal impairment. FORDHAM *et al.* (1965) were able to induce a marked decrease of osmolality in rats after 2 weeks, but they used a higher dose of phenacetin, and between 30 and 40 % of the rats died within 4 weeks. This suggests a high degree of general toxicity.

Somewhat surprisingly the dietary intake seems larger in the phenacetin rats than in the other groups. However, the phenacetin rats were more

restless than the others. The dose used may have given the stimulating effect also observed in humans taking large doses, whereas a depressant effect was noticed in experimental animals with higher doses (FORDHAM *et al* 1965 BERGLUND personal communication). However the weight gain in both drug groups was of similar magnitude as in the control groups. Thus, the difference in osmolality between the drug-fed rats and the controls can hardly be attributed to a protein deficiency in the drug-fed rats.

Another factor which might interfere with the concentration test is that the drugs per se might cause an osmotic diuresis (GILMAN 1965) However the urine osmolality was unchanged after one week of drug feeding and then a progressive decrease in osmolality occurred. Thus this could not reasonably be attributed to an osmotic load caused by the drugs, as this intake was unchanged throughout the experimental period.

The decreased urine osmolality during the 34th–41st weeks is consequently interpreted as indicative of renal tubular impairment. For further evidence exogenous vasopressin could have been administered.

The decrease in the concentrating capacity was of similar magnitude in the two drug series. In our previous study the osmolality was lower in the phenacetin (and NAPA) rats than in the acetylsalicylic acid animals. However in that study the concentration test was performed only once, and there was no test at the outset of the experiment.

After discontinuation of the drugs only a small number of rats were left for a follow-up study Most of the rats were sacrificed for morphological examination. The osmolality values were found to fluctuate and statistical evaluation was less satisfactory in the small test groups left, but it is evident that there was a tendency to rapid normalization of the osmolality This suggests that the concentration impairment was not caused by any severe morphological changes. Preliminary morphological studies have not shown any papillary necrosis or signs of infection in any of the groups.

Summary

The renal concentrating capacity was studied repeatedly in albino rats given large amounts of phenacetin or acetylsalicylic acid for 38–41 weeks. The dietary intake and weight curves did not indicate any general toxic effects. After 34 weeks, there was a significant decrease of renal concentrating capacity and this impairment tended to be rapidly reversible after discontinuation of the drugs. The decrease and the reversion were simultaneous and of similar magnitude for the two drugs.

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Effect of Epsilon Amino Caproic Acid on Adrenergic Nerve Function and Tissue Monoamine Levels

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By

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(Received August 14 1967)

Epsilon amino caproic acid (EACA) is a potent inhibitor of plasminogen activation (Okamoto *et al* 1957) and is therefore used clinically for the control of bleeding due to excessive fibrinolysis (for review see Nilsson, Andersson & Björkman 1966). Recently Lippmann Wishnick & Buyske (1965) and Lippmann & Wishnick (1965) reported that this substance reduced endogenous as well as exogenous noradrenaline (NA) levels in the heart of rats and dogs. The EACA-induced depletion of heart NA was prevented by bretylium and was markedly antagonized by a monoamine oxidase (MAO) inhibitor iproniazid. This indicated a similarity between the effects of EACA, reserpine and guanethidine

The fact that the pharmacological actions of EACA do not seem to have been extensively reported prompted this investigation In the present studies we have examined the effect of EACA on tissue monoamines, the functional significance of EACA-induced depletion of tissue NA and the interaction of EACA with a MAO inhibitor (pargyline), a membrane pump blocker (desipramine = DMI) and dexamphetamine. We have also examined the effects of a possible metabolite of EACA, *n*-amylamine.

Material and Methods

Biochemistry

Male Sprague-Dawley rats weighing 200–300 g were used. For doses, times and routes of administration, see Results. All doses in this study re calculated as the base. The rectal temperature was routinely monitored and, when necessary the animals were placed in warm room in order to prevent hypothermia. The rats were killed by cervical dislocation. The heart, the submaxillary and the sublingual salivary glands, the whole brain and the drenals were rapidly dissected out. The organs from two animals were pooled (

ice-cold 0.4 N perchloric acid (3–4 ml/gram tissue). The NA and adrenaline (A) were determined spectrophotofluorimetrically after cation exchange chromatography according to BERTLER, CARLSSON & ROSENGREN (1958), the dopamine (DA) according to CARLSSON & WALDECK (1958) and CARLSSON & LINDQVIST (1962), and the 5-hydroxytryptamine (5-HT), according to BERTLER (1961) and ANDÉN & MAGNUSSON (1967).

Adrenergic nerve functio

Blood pressure of unanaesthetized rats. The mean arterial pressure was recorded from the thoracic aorta of conscious Sprague-Dawley rats by means of indwelling catheters (POPOVIC & POPOVIC 1960). For this purpose a Statham Model P 23 Dc pressure transducer and a Grass Model 5 Polygraph were used.

Blood pressure f anaesthetized rats. Sprague-Dawley rats were naesthetized with urethane (1.2 g/kg s.c.) and a tracheal cannula was inserted. Drugs were injected into a jugular vein. The mean arterial blood pressure was recorded from the left carotid artery as described above. The rats were bilaterally adreno-demedullated about 3 weeks before the experiment (FARRIS & GRIFFITH 1949). Bilateral vagotomy in the neck was always performed.

Rat lower eyelid. Sprague-Dawley rats were anaesthetized with urethane (1.. g/kg s.c.) and prepared as in the blood pressure experiments. The cervical sympathetic on one side was isolated preganglionically and stimulated lectrically. Stimulation parameters are given below. The contractions of the rat lower eyelid to nerve stimulation were recorded semi-isometrically by means of a Grass Model FT-03 force displacement transducer and a Grass Model 5 Polygraph (OBIANWU 1967a).

Cat nictitating m mbrane. Cats of either sex weighing 1.5–3.0 kg were anaesthetized with pentobarbitone sodium (30 mg/kg i.p.). The cervical sympathetic trunk was dissected and divided about 5 cm caudal to the superior cervical ganglion. The cranial stump was placed on a bipolar platinum electrode and covered with liquid paraffin. The stimulation parameters are given under Results. The initial tension on the membrane was 2.0 g. The contractions of both membranes were recorded semi-isometrically by means of Grass Model FT-03 force displacement transducers and Grass Model 5 Polygraph.

Myocardial contractile force nd heart rate i cats. Cats weighing 1.8–3.5 kg were anaesthetized with pentobarbitone sodium (30 mg/kg i.p.). The chest was opened through a midsternal incision while respiration was maintained with a positive pressure pump. The right stellate ganglion was isolated and stimulated electrically as described under Results. A strain gauge arch, kindly supplied by Mr. Elof Gustafsson of AB Hässle, Göteborg, was sutured to the right ventricle. The myocardial contractile force was recorded on a Grass Model 5 Polygraph. Arterial blood pressure was measured from a femoral artery by means of a Statham Model P 23 Dc pressure transducer nd the Polygraph. By running the chart at a suitable speed (usually 5 mm/sec.) the heart rate was directly recorded. Bilateral cervical vagotomy was performed in all experiments. Body temperature was maintained at 37° with thermostatically regulated infrared lamp. Drugs were injected into a jugular vein or infused into a femoral vein.

Results

I. *Biochemistry*

Effect of graded doses of EACA on tissue NA levels. Six hours after the intraperitoneal administration of EACA (20 / m distilled water) in

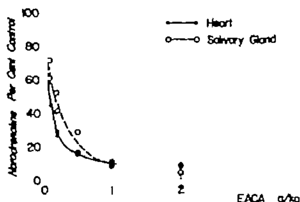


Fig. 1 Effect of various doses of epsilon amino caproic acid on the noradrenaline level in the rat heart and salivary glands. The drug was given intraperitoneally 6 hours before sacrifice. Each symbol represents one determination. Control values heart = 1.2 μg/g (s.e.m. = 0.18, n = 4), salivary glands = 1.4 μg/g (s.e.m. = 0.18, n = 4).

various doses, the rats were sacrificed and the hearts and salivary glands analyzed for NA. The results are presented in fig. 1 Increasing doses of EACA produced a progressive depletion of tissue NA, 2 g/kg causing almost complete depletion.

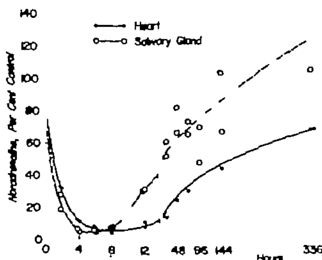


Fig. 2 Noradrenaline levels in the rat heart and salivary glands at various intervals after injection of epsilon amino caproic acid (2 g/kg i.p.). Each symbol represents one determination. Control values: heart = 1.0 μg/g (s.e.m. = 0.07 n = 6), salivary glands = 1.3 μg/g (s.e.m. = 0.09 = 6).



Fig. 3. Effect of epsilon amino caproic acid on the eyes of a conscious rat. To the left before the treatment. To the right 6 hours after n intraperitoneal injection of g/kg.

Tissue monoamine levels at various intervals after the administration of EACA. After the intraperitoneal injection of 2 g/kg EACA the NA contents of the rat heart and salivary glands were decreased to less than 10 / of the normal value at 6–8 hours (fig. 2). Recovery was slow in both organs. It required about 6 days in the salivary glands while it was not complete in the heart even after 14 days.

The levels of NA DA and 5-HT of the whole brain did not seem to be affected by EACA at any of the time intervals studied. The NA and A of the adrenal glands might have been somewhat reduced (about 30 %) at the earlier intervals but such a decrease is doubtful because of the large individual variations.

II. *Adrenergic nerve function*

Gross behaviour. Administration of EACA to rats produced obvious sympathomimetic effects such as piloerection, exophthalmos and mydriasis. After intraperitoneal injection of 2 g/kg these signs appeared within 15 min. and had usually disappeared after 2 hours. They were followed by progressive development of ptosis which seemed to be maximal after 4–8 hours (fig. 3). About 12–18 hours after the injection the animals appeared to be normal.

Results similar to those found in rats were observed in cats. After an intraperitoneal injection of 1 g/kg EACA an initial phase of sympathomimetic signs such as piloerection and mydriasis were seen. Thereafter the nictitating membranes were relaxed between 6 and 18 hours after the injection (fig. 4). Half this dose produced the same degree of relaxation of



Fig. 4. Effect of epsilon amino caproic acid on the nictitating membranes of a conscious cat. To the left before the treatment. T the right 4 hours after an intraperitoneal injection of 0.5 g/kg.

Table 1

Effect of epsilon amino caproic acid 2 g/kg i.p. on mean arterial blood pressure of unanaesthetized rats in per cent of the individual control value (means ± s.e.m. control value 121 mm Hg).

	Hours after administration							
	0	½	1	2	3	6	12	24
Blood pressure per cent control	100	126[1])	107	98	92	80[1])	91	97
s.e.m.	2.6	3.0	10.9	3.0	9.3	7.9	2.6	2.5
Number of experiments	12	10	11	12	12	10	8	8

[1]) Differs significantly ($P < 0.001$) from control value.

the membranes though the onset of the relaxation was quicker and the duration shorter.

No sedation was observed in the rats and cats at any time.

Blood pressure of conscious rats. The effect of EACA (2 g/kg i.p.) on the mean arterial blood pressure of conscious rats was studied in 12 experiments (table 1). During the initial sympathomimetic period the blood pressure was considerably increased. Thereafter the blood pressure fell to a minimum after about 6 hours. It had practically returned to the normal level 24 hours after the injection.

Blood pressure in anaesthetized rats. During anaesthesia the mean arterial blood pressure was also reduced (fig. 5). The increases in blood pressure in adreno-demedullated rats produced by carbachol hydrochloride (0.3 mg/kg) after atropine sulphate (10 mg/kg 10 minutes previously) were very small or absent after pretreatment with EACA (2 g/kg i.p. 6 hours previously) (fig. 5). The pressure response to tyramine hydrochloride (1 mg/kg) was also markedly antagonized though not as much as to carbachol (fig. 5). The response to (-)-NA-*d*-bitartrate was at least as great as in animals not pretreated with EACA (fig. 5).

Contraction of the rat lower eyelid to nerve stimulation. A slow intravenous injection of EACA (0.5–1 g/kg) in anaesthetized rats caused a marked contraction of the eyelid (fig. 6b) which persisted for up to 2 hours. In other rats pretreated with EACA (2 g/kg i.p.) 4–6 hours before the experiment, stimulation of the preganglionic cervical sympathetic elicited small or no responses of the eyelid (fig. 6c).

Contraction of the cat nictitating membrane to nerve stimulation. Like in the rat eyelid the cat nictitating membrane contracted after slow intra-



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Fig. 4. Effect of epsilon amino caproic acid on the nictitating membranes of conscious cat. To the left before the treatment. To the right 4 hours after an intraperitoneal injection of 0.5 g/kg.

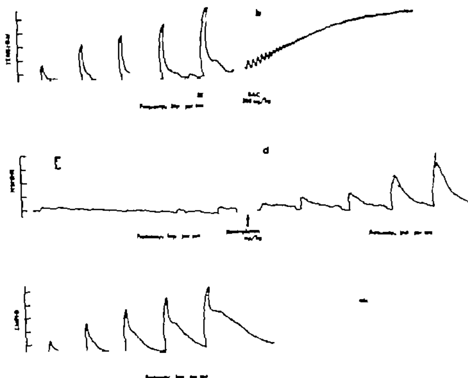


Fig. 6. Contractions of the rat lower eyelid. The cervical sympathetic was prepared and divided preganglionically. The cranial stump was stimulated with rectangular pulses (5 V 1 msec. for 10 sec. every 2 min. at the frequencies indicated). ( ) Stimulation responses in control rat. (b) Effect of infusion of epsilon amino caproic acid (EACA). (c) Stimulation responses in rat treated with EACA (2 g/kg i.p. 4 hours before the stimulation). (d) Effects of dexamphetamine (finished 60–45 min. before the stimulation) on the stimulation responses in the same rat as in (c) treated with EACA (2 g/kg i p. 5 hours before the stimulation). ( ) Effect of pretreatment with desipramine (10 mg/kg i.p. 30 min. before EACA) on the stimulation responses i rat treated with EACA (2 g/kg p. 5 hours before the stimulation).

The positive inotropic response to stellate ganglion stimulation was markedly antagonized ($P < 0.001$) (fig. 8c) the increase in contractile force was reduced from 67 (s.e.m. = 6 n = 6) to 17 (s.e.m. = 7 n = 6) per cent. The positive chronotropic response to ganglion stimulation was, however only insignificantly reduced by EACA. The positive inotropic and chronotropic responses to ( )-NA-*d*-bitartrate and ( )-isoprenaline-*d*-bitartrate were, however not reduced after pretreatment with EACA (fig. 8a and b).

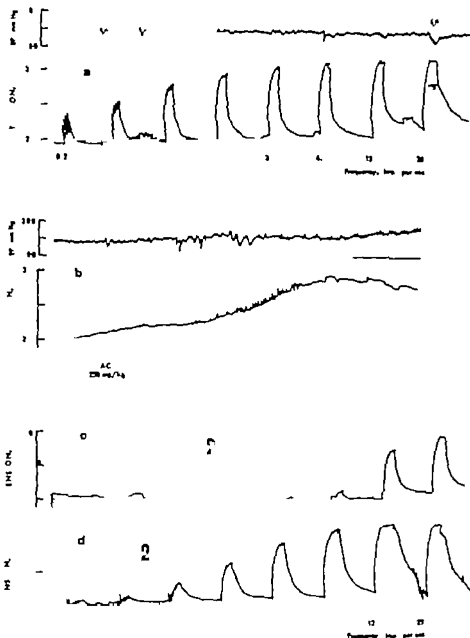


Fig. 7. Contractions of the cat nictitating membrane. The cervical sympathetic was prepared and divided preganglionically. The cranial stump was stimulated with rectangular pulses (6 V, 2 msec. for 30 sec. every 2 min.). (a) Stimulation responses in a control cat. (b) Effect of infusion of epsilon amino caproic acid (EACA). (c) Stimulation responses in a cat treated with EACA (0.5 g/kg i.p. 5 hours before the stimulation). (d) Effect of infusion of dexamphetamine (0.5 mg/kg i.v. finished 60–45 min. before the stimulation) on the stimulation responses in the same cat as in (c) treated with EACA (0.5 g/kg i.p. 6 hours before the stimulation).

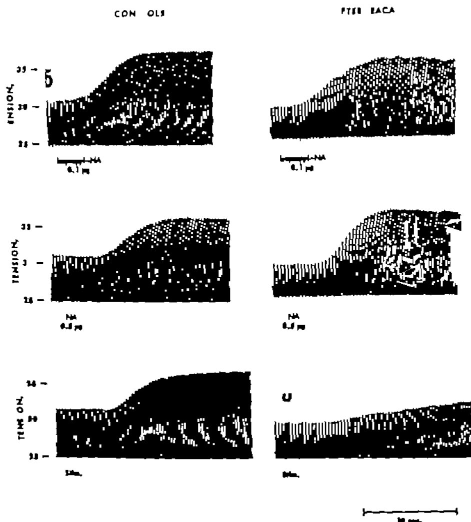


Fig. 8. Myocardial contractile force and heart rate in open-chest cats. To the left control cat. T the right cat treated with epsilon amino caproic acid (0.5 g/kg i p 4 hours before the experiment). The noradrenaline (NA) and isopropyl-NA were injected in jugular vein. The right stellate ganglion was prepared and stim lated with rectangular pulses (10 V 2 msec. for 15 sec. at 10 impulses per sec.).

III *Effects of pargyline desipramine and amphetamine on the biochemical and functional actions of EACA*

Pretreatment of rats with the MAO inhibitor pargyline hydrochloride (75 mg/kg i.p) 1 hour before injection of EACA (2 g/kg i.p) greatly anta gonized the EACA-induced loss of NA in the heart (table 2). After the

Table 2

Levels of noradrenaline (μg/g; mean ± s.e.m.) in the rat heart and salivary glands after the treatments listed in the left column. Number of experiments is indicated in brackets.

Treatment (Dose time before sacrifice)	Noradrenaline concentration: Heart		Noradrenaline concentration: Salivary glands	
No drug treatment	1.03 ± 0.069	(7)	1.37 ± 0.084	(7)
EACA (2 g/kg i.p., 6 hr)	0.08 ± 0.008	(8)	0.11 ± 0.018	(8)
Pargyline (75 mg/kg i.p., 7 hr)	1.12 ± 0.043	(5)	–	
Pargyline + EACA (75 mg/kg i.p. 7 hr) + (2 g/kg i.p., 6 hr)	0.66 ± 0.077	(5)	–	
DMI (10 mg/kg i.p., 6½ hr)	0.99 ± 0.064	(4)	1.13 ± 0.130	(4)
DMI + EACA (10 mg/kg i.p., 6½ hr) + (2 g/kg i.p. 6 hr)	0.77 ± 0.053	(10)	0.78 ± 0.054	(10)
Dexamphetamine (5 mg/kg i.p. 6½ hr)	0.75 ± 0.040	(6)	0.91 ± 0.164	(6)
Dexamphetamine + EACA (5 mg/kg i.p. 6½ hr) + (2 g/kg i.p. 6 hr)	0.16 ± 0.013	(11)	0.14 ± 0.016	(10)
EACA + Dexamphetamine (2 g/kg i.p. 6 hr) + (5 mg/kg i.p. ½ hr)	0.05 ± 0.008	(6)	0.15 ± 0.033	(6)

pargyline pretreatment, the sympathomimetic signs such as exophthalmos and piloerection appeared after a shorter latent period and were considerably more pronounced than in animals not pretreated. In the pargyline pretreated rats this effect was not followed by any signs of adrenergic blockade such as ptosis.

Pretreatment of rats with DMI hydrochloride (10 mg/kg i.p.) 30 minutes before injection of EACA (2 g/kg i.p.) almost completely prevented the EACA induced disappearance of NA from the heart and salivary glands (table 2). After this pretreatment neither the initial sympathomimetic signs nor the ptosis were observed following EACA injection. Moreover the contractions of the rat lower eyelid to electrical sympathetic stimulation were about the same as in untreated animals except that the relaxation of the eyelid following cessation of stimulation was slower as is usually the case after DMI treatment (fig. 6e). In addition the relaxation of the cat nictitating membrane after injection of EACA (0.5 g/kg i.p.) was prevented by pretreatment with DMI (2 mg/kg i.p. 30 minutes before EACA).

If dexamphetamine-d-bitartrate (5 mg/kg i.p.) was given to rats 30 minutes before EACA (2 g/kg i.p.) the NA of the heart and salivary glands were greatly reduced while in the heart it was about twice as high as after treatment with EACA only (table 2). This pretreatment with dexamphetamine partially prevented the appearance of ptosis after EACA treatment. If dexamphetamine (5 mg/kg i.p.) was given 5½ hours after EACA (2 g/kg i.p.) the ptosis rapidly disappeared and was followed by exophthalmos. In the stimulation experiments too it was found that infusion of dexamphetamine partially restored the responses of the rat eyelid (fig. 6d) and the cat nictitating membrane (fig. 7d) to sympathetic stimulation.

IV *Biochemical and functional effects of n-amylamine*

LIPPMANN, WISHNICK & BUYSKE (1965) and LIPPMANN & WISHNICK (1965) studied the effects of compounds structurally related to EACA on the NA levels in the rat heart and described some structural requirements for depletion. However *n*-amylamine was not included in their investigations. This compound may be a metabolite of EACA formed by decarboxylation. Therefore, *n*-amylamine (0.04 g/ml) was administered subcutaneously to rats in repeated doses of 0.1–0.2 g/kg at 2 hour intervals, up to a total dose of 1 g/kg. Following each injection this substance for about 1 hour produced strong sympathomimetic effects similar to those described for EACA. Subsequent injections elicited less pronounced sympathomimetic effects. The NA concentrations of the heart and salivary glands were reduced to 10–20 % of the normal, 2 hours after the last dose. Throughout the investigation no signs of impaired adrenergic function such as ptosis were observed in the *n*-amylamine treated rats, although some rats were studied for 12 hours after the last injection.

Discussion

The initial sympathomimetic signs after EACA injection have been described previously (SPINK & VICK 1961, RAMOS, CHAPMAN, CORRADO & FORTES 1961, CUMMINGS & WELTER 1966). It is likely that an increased release of NA from the sympathetic nerve terminals is responsible for these effects since they are not observed when the NA stores have been depleted by reserpine (CUMMINGS & WELTER 1966). Further evidence for an indirect sympathomimetic action of EACA is the present finding that the effects were abolished by DMI and were increased by a MAO inhibitor

In the present study it was also shown that the sympathomimetic phase was followed by a blockade of the sympathetic nerve functions. Since the responses to postganglionic sympathetic stimulation and to tyramine were reduced though not that to NA, the blockade is in all likelihood due to a reduced NA release from the postganglionic, sympathetic nerve terminals and not to a ganglionic blockade or an adrenergic receptor blockade. The impaired adrenergic transmission probably explains the orthostatic hypotension and the diarrhoea which are common side effects when EACA is used as an antifibrinolytic agent in humans (see NILSSON ANDERSSON & BJÖRKMAN 1966).

In addition to changing the NA release from the sympathetic nerves EACA also caused a severe loss of NA from the sympathetically innervated organs This depletion was first described by LIPPMANN WISHNICK & BUYSKE (1965) and LIPPMANN & WISHNICK (1965) who showed a dose response curve and a time course similar to those reported in this paper Furthermore no clearcut reductions of the monoamines in the brain and in the adrenals were found in these investigations. Similar results have also been obtained by GRANSTRAND LINDGREN NYBÄCK & SEDVALL (1966) The latter investigators also reported a poor penetration of EACA into the brain which probably explains the absence of any central effects.

Studies on the metabolism of EACA have not disclosed an active metabolite and most of the drug is rapidly excreted in the urine (NILSSON SJOERDSMA & WALDENSTRÖM 1960 MCNICOL, FLETCHER, ALKJAERSIG & SHERRY 1962 MELANDER GLINIECKI, GRANSTRAND & HANSHOFF 1965). The absence of sympathetic blockade after *n*-amylamine injections indicates that this substance is not an active metabolite of EACA. The loss of NA after this treatment was of such a magnitude that a similar reduction of NA stores after EACA administration was accompanied by ptosis in rats.

The actions of EACA on the adrenergic transmission are probably dependent on a concentration of the compound in the sympathetic nerves as suggested by the following facts. After unilateral, chronic, postganglionic sympathectomy of rat submaxillary glands, a smaller amount of ^{3}H EACA accumulates in the denervated than in the contralateral innervated glands (OBIANWU 1967b) The uptake of ^{3}H EACA by the sympathetically innervated organs is also reduced by pretreatment with DMI or protriptyline (OBIANWU 1967b STITZEL, LUNDBORG & OBIANWU 1967) These drugs block the amine pump located at the level of the cell membrane of the adrenergic neurones (MALMFORS 1965 CARLSSON & WALDECK 1965a). These observations may explain why EACA was ineffective on both the adrenergic nerve function and the NA levels after DMI pretreatment.

The poor action of EACA on the adrenal medulla may be due to a negligible concentration of the substance at this site, since the described amine membrane pump is probably weakly developed in the catecholamine cells of this organ. The ineffectiveness of EACA to lower the adrenal catecholamines may also partly be explained by the absence of central effects, since part of the adrenal depletion after reserpine treatment is explained by a centrally induced increase of the impulse flow in the splanchnic nerves.

In subcellular distribution studies, it has been observed that during the 4 hours following an injection of ^{3}H EACA there is a progressive increase in the amount of EACA found in the particulate fraction of the mouse heart with a concomitant decrease in the supernatant content (STITZEL, LUNDBORG & OBIANWU 1967). This finding indicates that EACA is slowly incorporated into the storage granules after having been taken up into the adrenergic neurones by the membrane pump

EACA does not only use the dual amine uptake-concentration mechanism in the adrenergic neurones but also seems to inhibit its two components. These blockades have been demonstrated by means of injection of ^{3}H-metaraminol (OBIANWU 1967c). Pretreatment with EACA causes a clearcut reduction of the ^{3}H metaraminol accumulated in the rat heart in 30 minutes. A similar effect is produced by DMI whereas reserpine is almost ineffective during this short time. During the following 2½ hours, EACA reduces the level of ^{3}H-metaraminol retained to about the same extent as reserpine whereas DMI induces only a slight further decrease. The diminution in ^{3}H-metaraminol between 30 min. and 3 hours indicates that EACA acts like reserpine, i.e. inhibits the incorporation of amines in the storage granules The observations that there are reductions in the *in vitro* uptake of ^{14}C adrenaline by adrenal medullary granules in the presence of EACA and in the *in vivo* uptake of ^{3}H-α methyl NA by the particulate heart fraction of EACA-treated mice, are also consistent with such a view (STITZEL, LUNDBORG & OBIANWU 1967) Furthermore, EACA injection induces a loss of ^{3}H-metaraminol from the particulate fraction of the mouse heart (STITZEL, LUNDBORG & OBIANWU 1967).

The partial prevention of the EACA induced ptosis by pretreatment with dexamphetamine is somewhat surprising since dexamphetamine hardly inhibited the EACA-induced disappearance of NA Thus, dexamphetamine does not significantly change the EACA uptake by the sympathetic nerves and differs in this respect from the membrane pump blocking agents. It is also noteworthy that dexamphetamine produced strong sympathomimetic effects and partially restored the sympathetic nerve stimulation response in the EACA treated rats with very low levels of

NA No explanation of these remarkable interactions between EACA and dexamphetamine can be given till more information has been obtained about the pool from which dexamphetamine releases NA

The actions of EACA are very similar to those of guanethidine and in some respects also to those of reserpine Firstly EACA and guanethidine (SHEPHARD & ZIMMERMAN 1959 CASS, KUNTZMAN & BRODIE 1960) produce a very marked NA disappearance from the sympathetically innervated organs but not from the adrenals or from the brain. Reserpine, on the other hand causes a depletion of monoamines from all organs. Secondly EACA and guanethidine (STONE, PORTER, STAVORSKI, LUDDEN & TOTARO 1964 CARLSSON & WALDECK 1965a) need the cell membrane pump of the sympathetic neurones to influence the NA stores and the adrenergic transmission. Reserpine, however enters the neurones even after membrane pump blockade by DMI (CARLSSON & WALDECK 1965a). This difference is probably due to low and high lipid solubility respectively Such a difference in lipid solubility should also influence the ability to pass the blood-brain barrier and thus, explain why EACA and guanethidine seem to lack any central effects. EACA and guanethidine but not reserpine also block the uptake of amines at the level of the cell membrane of the adrenergic neurones (LINDMAR & MUSCHOLL 1964 CARLSSON & WALDECK 1965b MALMFORS 1965 SHORE & GIACHETTI 1966 OBIANWU 1967c LUNDBORG & STITZEL 1967) Such an action in combination with release of NA from the storage granules to the cytoplasm may be responsible for the initial sympathomimetic actions seen after EACA and guanethidine but not usually after reserpine. After pretreatment with a membrane pump blocking agent, however reserpine also produces sympathomimetic effects Thirdly EACA like guanethidine (LINDMAR & MUSCHOLL 1964 SHORE & GIACHETTI 1966 OBIANWU 1967c LUNDBORG & STITZEL 1967) and reserpine (KIRSHNER 1962 CARLSSON HILLARP & WALDECK 1963) block the incorporation of NA and other amines into the storage granules. EACA and guanethidine (CHANG, COSTA & BRODIE 1965) are also taken up by the granules. All three drugs can also block the responses to sympathetic nerve stimulation and to tyramine. This action of reserpine seems to be produced by the effect described on the granules the sympathetic nerve function ceases only when the NA stores are severely depleted (GAFFNEY CHIDSEY & BRAUNWALD 1963) and returns simultaneously with a marked recovery of the uptake function (ANDÉN & HENNING 1966) The sympathetic blockade after guanethidine starts, however before any significant reduction of the NA is detectable (CASS & SPRIGGS 1961 GAFFNEY CHIDSEY & BRAUNWALD 1963). Therefore it has been speculated that guanethidine may interfere with the ability of the nerve impulses to influence an important pool

of the NA store. After EACA treatment the time correlation between the NA loss and the sympathetic nerve impairment has not been studied very carefully It appears, however that the adrenergic transmission failed only when more than 60 % of the NA had been lost and that it recovers concomitantly with a small rise of the NA levels. Fourthly many investigations have demonstrated that the guanethidine-induced adrenergic transmission blockade can be antagonized by amphetamine like substances given before or after guanethidine (see DAY & RAND 1963 CHANG COSTA & BRODIE 1965). In this respect EACA seems to resemble guanethidine except that amphetamine could hardly prevent the NA disappearance after injection of EACA (cf CHANG COSTA & BRODIE 1965) After reserpine treatment amphetamine also appears to be able to restore partially the response to nerve stimulation (DAY 1962, OBIANWU 1967d).

Summary

Epsilon amino caproic acid (EACA) injection in rats (0 5–2 g/kg) and in cats (0.25–1 g/kg) produced during the first few hours sympathomimetic effects due to an increased noradrenaline (NA) release. Thereafter *the adrenergic transmission was blocked presynaptically for 10–16 hours.* In rats the NA of sympathetically innervated organs was reduced by EACA (2 g/kg i.p.) to less than 10 % in 6–8 hours and after 12 hours started to return slowly The monoamines of the brain and adrenal medulla were not significantly lowered The membrane pump blocking agent desipramine prevented both the sympathomimetic and the sympatholytic effects of EACA and greatly antagonized the NA depletion. After monoamine oxidase inhibition by pargyline, EACA induced only strong sympathomimetic signs and a small NA loss. Pretreatment with amphetamine partially inhibited the sympatholytic actions of EACA but not the disappearance of NA. After severe NA depletion by EACA *amphetamine partially* restored the responses to sympathetic nerve stimulation. The sympathomimetic effect is probably due to a blocking of the NA uptake by the granules as well as through the cell membrane. The adrenergic transmission blockade may be due, like after reserpine, to the NA depletion but a guanethidine-like effect may also contribute to this.

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The Effect of Ethanol on the Gastrointestinal Absorption of Drugs in the Rat

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Ethanol is known to enhance the effect of many drugs particularly those with a central depressant action In recent years the ethanol-drug interactions have become a more and more serious problem, not only because of the increased and often indiscriminate use of central depressant drugs, but also because of the increased demand for unimpaired coordination and judgment, required for the safe operation of automobiles and other complex machinery Under the direction of Professor K. SOEHRING the Deutsche Gesellschaft für Verkehrsmedizin has had annual meetings in Bad Oeynhausen since 1960, dealing particularly with this topic (SOEHRING 1960). Pharmacodynamic interactions between ethanol and drugs have been investigated both experimentally and clinically though, there is still some controversy about the nature of the observed synergism. This question has recently been reviewed by ZIPF & HAMACHER (1967).

Previously only a few experiments were made on the effect of ethanol on drug absorption and this has always been determined by the drug concentrations reached in the blood after oral administration. As these experiments have given contradictory results (GRAHAM 1960 MELVILLE *et al* 1966) it seemed worthwhile to study the effect of ethanol on the gastrointestinal drug absorption by a different method. It was decided to study the absorption from the stomach and the small intestine separately using techniques for measuring the absorption by the disappearance of drug from solutions introduced into or passing through the stomach and the small intestine respectively The effect of ethanol was studied when both drugs were administered together or when ethanol was already present in

the blood at the time of drug absorption. After the beginning of this study GREBER & SOEHRING (1967) and SEIDEL (1967) showed that ethanol is capable of influencing the distribution of some barbiturates and SCHÜPPEL (1967) found, that ethanol is capable of delaying the metabolism of aminophenazone and phenazone. These experiments only further indicate the advantage and in the present ethanol studies the necessity of measuring the disappearance rate of the drug from the site of absorption.

Methods

All experiments were made on female rats of the Leo strain, weighing 180–220 g. Before the experiments, the rats were fasted for about sixteen hours but given water ad libitum.

Absorption from the stomach. Under urethane anaesthesia (1,2 g/kg intraperitoneally) the cardia was first ligated and then the pylorus around a short polyvinyl cannula equiped with a stopcock. Drug absorption per hour from 0 01 N HCl was measured according to the method described by SCHANKER *et al* (1957). After washing the gastric lumen with saline and three times with the drug solution warmed to body temperature, 10 ml of the drug solution were thoroughly mixed with the contents of the gastric lumen by means of syringe and 5 ml of this solution left in the stomach. The remaining 5 ml, and after one hour the solution in the stomach, were used for the determination of initial and final drug and phenol red concentrations and for measuring the pH. The per cent absorption per hour was calculated by the following equation

$$\text{Per cent absorbed in one hour} = 100 - 100\left(\frac{\text{final drug conc.}}{\text{initial drug conc.}} \times \frac{\text{initial phenol red conc.}}{\text{final phenol red conc.}}\right)$$

According to SCHANKER *et al.* (1957) 98 ± 2 per cent of phenol red was recovered from the stomach after one hour so that variations in the concentrations of this dye can be used for the correction of volume changes during the experiments. In addition to per cent absorption, the volume correction factors were calculated (initial phenol red conc./final phenol red conc.) to detect dilution (factor > 1) or concentration (factor < 1) during the experiments. In experiments with promethazine (200 μg/ml), this interfered with the determination of phenol red because of precipitation in the basic milieu. Thus in these experiments the mean volume correction factors found in the corresponding experiments with phenobarbital (phenemal NFN) and pentobarbital (mebumal NFN) were used. The drug solutions always contained 200 μg/ml of the drug concerned (calculated as the base or the acid), 10 μg/ml phenol red, and 0 9 % NaCl in 0 01 N-HCl. In one series of experiments, ethanol was present in the drug solutions in concentrations ranging from 1 20 % (w/v). In another series, a blood ethanol concentration of approximately 1.5 mg/ml was maintained during the experiment. After a priming dose of 1.05 g/kg given intravenously (10 % (w/v) ethanol solution) an ethanol infusion of 0.5 g/kg/h (infusion rate 0.02 ml/min.) was started into the femoral vein. When 30 minutes had elapsed to obtain equilibrium, a 50 μl blood sample was taken from the carotid artery for determination of the initial blood ethanol concentration, and the absorption during the next hour determined. After this, a second blood sample was taken for the determination of the final blood ethanol concentration. In control experiments, ethanol was substituted by the same volume of 0.9 % saline.

Absorption from the small intestine. The single perfusion method described by SCHANKER *et al.* (1958) was used with minor modifications. Urethane (1,2 g/kg intraperitoneally) was used as anaesthetic. The bile duct was not ligated. Perfusion of the small intestine was carried out by means of a Braun infusion pump at a rate of 1.0 ml/min., which gave a

passage time of about ten minutes. The intestine was cleared by perfusion with the drug solution (37) fo 30 minutes, and then three 10 minutes samples were collected The relative rate of absorption was calculated by means of the equation above and the drug and phenol red concentrations entering (initial) and leaving (final) the intestine. As in the experiments on the stomach, olume correction factors were calculated and the pH measured in all solutions. Only experiments in which samples had a volume of 8.5–11.5 ml were used in the results. As there was no difference in absorption rate in the first, second and third samples, the given rates are calculated from all samples together so that the number of determinations (n) is three times the number of animals used. The perfusion solutions contained the appropriate drug in a concentration of 200 µg/ml (pentobarbital and promethazine 50 µg/ml, in rder to avoid any pharmacological effects of these drugs), the values being calculated as the base or the acid, NaCl 137 mM KCl 2.68 mM CaCl 1.80 mM phenol red 10 µg/ml the pH was adjusted to 6.0 with HCl or NaOH. In one series of experiments ethanol was present in the drug solutions in concentrations of 0.5 1.0 or 2.0 % (w/) In another series, after a priming dose of 1.05 g/kg intravenously the intravenous ethanol infusion (0.5–0.8 g/kg/h) and the perfusion of the intestine were started simultaneously After the prel minary period of 30 minutes, an initial blood sample was taken (50 µl from the carotid artery) after which the three 10 minutes samples of perfusate were collected and then a final blood sample was taken for ethanol determination. In control experiments ethanol was substituted by the same volume 0.9 % saline.

Body temperature during the in i xperiments. The body temperature was followed in some of the experiments on intestinal bsorption. After preparation the rectal temperature of the rats was 34–35 Independent of the presence f ethanol the temperature never fell below 33 at the end of the experiment (i.e. one hour later).

Absorptio i vitro. Rats were killed by a blow on the neck and two 8.5 cm segments of each jejunum were used, taken 10 cm from the pylorus. The segments were placed in and washed by a gentle stream of Tyrode (37°). Cannulas were tied into the upper and the lower end of the segments nd the preparations placed i organ baths, containing 8.5 ml of Tyrode, bubbled through with carbogen (4% CO_2 and 96 % O_2) Perfusion in the caudad direction with the drug solution (37°) at a rate of 0.5 ml/min. was started immediately After a preliminary period of 10 mi utes, the Tyrode was removed by suction, the bath and the organ were washed twice with Tyrode, and 8.5 ml f fresh Tyrode placed in the bath. After 30 minutes the Tyrode was removed for analysis. The intestinal segments were dried overnight and weighed. Results were presented as the amount of drug found in the 8.5 ml f Tyrode per 100 mg of intestine (the segments weighed about 70 mg). The Tyrode contained the following salts in mM N Cl 137 KCl 2.68 $CaCl$ 1 80, $NaHCO_3$ 11.90, NaH_2PO 0 41 nd 0.1 % glucose. The pH in this solution when bubbled with carbogen is about 7 4 The drug solutions contained Phenol red 10 µg/ml and pentobarbital 1 mM or sulphaguanidine 400 µg/ml in 1/15 M sodium phosphate buffer pH 5.3 The pH value of 5.3 was chosen since HOGBEN *et al.* (1959) found this to be the "virtual pH" at the intestinal epithelial surface Phenol red was given so as to detect any leakage in the preparation. The effect of ethanol was determined when present on the serosal side (in the Tyrode 1.5 mg/ml) or on the mucosal side (in the drug solution 1.0 or 2.0 % (w/v)) of the intestine Since SMYTH & T YLOR (1957) have stressed the importance of adequate oxygenation of the mucosa in experiments on active absorptive processes, some control experiments were done in which the testine was perfused with pentobarbital soluted in Tyrode saturated with carbogen (pH 7 4). In these experiments, there was a trend to slightly higher bsorption than in those seen in table 6 however this was not influenced by the presence of ethanol (1.5 mg/ml in the bath or 2.0 % (w/v) in the perfusion fluid respectively).

Excretion of neutral red int the stomach. These experiments were made in order to get

an estimate of the blood circulation to the gastric mucosa (SCHANKER *et al* 1957). Rats were prepared and a steady state blood ethanol level of 1.5 mg/ml was maintained as usual. 5 mg/kg neutral red, a dose with no effect on the blood pressure, was injected into the femoral vein and the amount of neutral red excreted in 5 ml of 0.01 N HCl in 0.9 % NaCl, placed in the ligated stomach, was determined one hour after the injection of the dye.

Gastric acid secretion. In order to get an estimate of the effect of ethanol on the gastric acid secretion, this was determined in rats under urethane anaesthesia (1.2 g/kg intraperitoneally), in which the cardia and the pylorus had been ligated. One hour after ligation, the abdominal incision was reopened, the stomach removed, the content poured out and the residue washed down with 10 ml of distilled water into a beaker. The acid content was determined by potentiometric titration to pH 8.0 with 0.1 N NaOH.

Analytical methods. All quantitative determinations were carried out by spectrophotometry.

Ethanol was determined by the ADH-method (modification 1) described by LUNDQUIST & WOLTHERS (1958). Alcohol dehydrogenase (from yeast) and DPN (NAD) were obtained from C. F. Boehringer & Soehne G.m.b.H., Mannheim, Germany. In the determinations of the drug solutions, the precipitation step with perchloric acid was omitted. All blood ethanol concentrations indicated were determined in arterial blood.

Barbiturates, i.e. barbital (diemal NFN), pentobarbital (mebumal NFN) and phenobarbital (phenemal NFN), were determined by the method of FREY *et al* (1961). Suitable amounts of the drug solutions containing the appropriate barbiturate were added to 1 ml of 1.5 M NaH_2PO_4, extracted with chloroform and reextracted into 4.00 ml of 0.45 N NaOH. The extinction difference between 255 and 275 mμ was taken as a measure of the barbiturate content.

Neutral red. Solutions were made alkaline with 1 N NaOH, extracted with 4 volumes of chloroform. The chloroform was evaporated to dryness, the residue taken up in 0.5 ml of 0.01 N HCl and the extinction measured at 535 mμ in a microcuvette. Recovery was found to be approximately 80 %.

Phenol red. Samples containing phenol red were appropriately diluted with NaOH and their extinction measured at 550 mμ (SCHANKER *et al.* 1957).

Promethazine. The method was based on that of BURGER & BERNDNER (1958). A suitable amount of the solution containing promethazine was added to 0.5 ml of 0.1 N NaOH, extracted with 20 ml of n-hexan, and reextracted into 4.00 ml of 0.5 N-HCl. The extinction difference between 250 and 280 mμ was taken as a measure of the promethazine concentration. Recovery was 95 %.

Sulphaguanidine. BRATTON & MARSHALL's method (1939) was used. 50 μl of drug solution was added to 4.00 ml of distilled water (in *in vitro* experiments 4.00 ml of Tyrode was taken), and 700 μl of 4 N-HCl added. Subsequently the reagents were added as follows: 0.5 ml of sodium nitrite (0.1 %). After standing for three minutes 0.5 ml of ammoniumsulphamate (0.5 %) and after standing for two minutes 0.5 ml of N-(1-naphthyl)-ethylene diamine dihydrochloride (0.1 %) were added. The extinction at 540 mμ was taken as a measure of the sulphaguanidine content.

Statistical methods. Results are presented as the arithmetical mean (m) ± standard deviation (s). Only in the excretion of neutral red and gastric acid secretion were the geometrical means used.

pKa-values of the drugs used. Barbital 7.8 (BRODIE & HOGBEN 1957), pentobarbital 8.1 (BRODIE *et al* 1960), phenobarbital 7.2 (WADDELL & BUTLER 1957), promethazine 8.6 (PHARMACOPOEA NORDICA, EDITIO DANICA 1963, vol. II, p. 5 4), sulphaguanidine. One very weak acidic group pKa > 10 and two very weak basic groups pK 2.75 and 0.5 (BRODIE *et al.* 1960).

Results

1 *Stomach*

Simultaneous application of drug and ethanol

The results of the experiments on absorption and the effect of ethanol, when present in the stomach, are summarized in table 1 In some experiments the blood ethanol concentrations were determined at the end of the

Table 1

Absorption from the rat stomach, and the effect of simultaneous administration of drug and ethanol.

The per cent absorbed in one hour is expressed as the mean ± standard deviation. The P values are calculated by means of Student's t-test.

Drug		Ethanol concentration (w/v) in the stomach: 0	1	2.5	5	10	15	20
		Per cent absorbed in one hour						
Phenobarbital	m	17 1	20.7	20.1	24.8	22.3		
	s	±4 7	±2.4	±3 4	±4 7	±5.6		
	n	(18)	(12)	(12)	(18)	(12)		
	P		<0.5		<.001	< 02		
Pentobarbital	m	23 7	24.4	31 7	30.1	28.0	25.3	21 4
		±3.8	±3.6	±3 1	±4.5	±6.2	±6.9	±7 1
	n	(22)	(10)	(10)	(9)	(16)	(9)	(16)
	P			< 001	<.001	<.02		
Promethazine	m	−0.2			−0.9	0.7		
	s	±3.2			±3 6	±5 8		
	n	(8)			(7)	(6)		
Ethanol	m	37 7[1]	50.3	48.0	46.1	46.8	33.5	34.8
	s	±8.6	±6.6	±4 1	±4.3	±11.8	±5.6	±11.0
	n	(6)	(12)	(10)	(9)	(6)	(4)	(12)
	P		<.01	<.01	<.05			
Final blood ethanol[2]	m	[3]	[3]	0.3	0.8	2.2	2.0	2 0
				±0.02	±0.14	±0.09	(1.5–3 0)	(1.3–2.8)
	n			(6)	(6)	(6)	(6)	(6)

[1]) In control experiments the concentration of ethanol was 200 μg/ml in the stomach.

[2]) Arterial blood ethanol concentrations (mg/ml) at the end of some of the experiments. With 15 and 20 ethanol, the highest and lowest blood ethanol found, is given instead of s.

[3]) Not detectable.

Table 2

Absorption from the rat stomach, and the effect of a steady blood ethanol concentration. The per cent absorbed in one hour is expressed as the mean ± standard deviation. Number of experiments are indicated in brackets. The P-values are calculated by means of Student's t-test.

Drug	Per cent absorbed in one hour		P	Blood ethanol conc. mg/ml ± s	
	Control	Treated		Initial	Final
Phenobarbital	13.8 ± 2.5 (8)	19.4 ± 2.1 (7)	<.001	1.7 ± 0.05	1.8 ± 0.10
Pentobarbital	20.8 ± 3.6 (12)	26.0 ± 4.2 (13)	<.01	1.5 ± 0.06	1.6 ± 0.11
Promethazine	−1.2 ± 2.8 (6)	0.2 ± 3.8 (5)		1.5 ± 0.08	1.6 ± 0.11

experiments. These are indicated at the bottom of table 1. As can be seen, the absorption of the two barbiturates was significantly enhanced by ethanol concentrations ranging from 1–10 / (w/v), but was depressed again with higher concentrations. A similar pattern can be seen with ethanol itself. Contrary to the barbiturates, promethazine which is completely ionized at the pH of the stomach content, is not absorbed neither with nor without ethanol in the stomach.

Absorption during a steady blood ethanol concentration

In table 2 the effect of a steady state blood ethanol concentration on the absorption is shown. The concentration of about 1.5mg/ml corresponds to that reached one hour after the introduction of 5 ml of 5 to 10 / (w/v) ethanol solutions into the stomach (table 1). Here too ethanol significantly enhanced the absorption of the two barbiturates but there was no effect on the absorption of promethazine.

Pathological changes, pH and volume variations in the stomach under the influence of ethanol

Ethanol is known to cause irritation of organic tissues. In concentrations exceeding 10 / (w/v) in the stomach, this proved to be so strong, that marked congestion and even very often ulceration with exudation of blood occurred. These changes were paralleled by a marked rise in the pH and volume correction factors, as summarized in table 3. In experi-

Table 3

pH and volume variations in the stomach under the influence of ethanol

Initial pH (n = 185) [1] 2.06 ± 0.04

Ethanol concentration (w/v) in the stomach

	0	1	2.5	5	10	15	20
	Final pH[1]						
m	2.37	2.50	2.62	3.01	4.02	6.44	6.87
s	±0.20	±0.26	±0.22	±0.44	±0.86	±0.24	±0.41
n	(48)	(22)	(22)	(34)	(34)	(9)	(16)
	Volume correction factors[2]						
m	1.05	1.06	1.06	1.06	1.08	1.13	1.17
s	±0.02	±0.0	±0.02	±0.02	±0.04	±0.11	±0.12
n	(40)	(22)	(22)	(27)	(28)	(9)	(16)

[1] From experiments with phenobarbital pentobarbital and promethazine (table 1).
[2] From experiments with phenobarbital and pentobarbital (table 1).

ments with a steady state blood ethanol level, no such rise in the pH and volume correction factors was observed The changes were the same as those in the control experiments, shown in table 3

Influence of ethanol on the circulation in the gastric mucosa

In trying to explain the effects of ethanol on the absorption of drugs from the stomach, the blood circulation through the gastric mucosa is of great interest. Since a direct determination is very difficult in the rat, this was estimated indirectly by means of the excretion of neutral red into the stomach after intravenous administration (SCHANKER *et al* 1957) This amounted to 2.7 μg/h (1 8–4 0 n = 6) in the controls and 4 3 μg/h (2.6–7.2, n = 6) in rats in which a blood ethanol concentration of 1 5 mg/ml was maintained during the experiment. These results were taken as evidence of an enhanced blood supply to the gastric mucosa caused by the ethanol a fact which is also suggested by RITCHIE (1965) To be certain, however that the increased excretion of neutral red into the stomach in the ethanol treated animals, was not caused by an augmented gastric acid secretion under the influence of ethanol, the latter was determined and found to be 21 0 (13 8–31 9 n = 6) μeq HCl/h in control experiments and 18.2 (7.3–45 0, n = 6) μeq HCl/h in the treated animals ($p > 0.05$).

Table 4

Absorption from the rat small intestine perfused with aquous or ethanolic drug solutions. The results are expressed as the mean ± standard deviation. Number of observations (i.e. 3 times the number of animals used) are indicated in brackets. The P-values are calculated by means of Student's t-test.

Drug	Ethanol concentration (% w/v) in small intestine			
	0	0.5	1.0	2.0
	Per cent absorbed within 10 minutes			
Barbital	43.1 ± 5.3 (18)	44.5 ± 4.8 (18)	41.0 ± 4.7 (15)	45.8 ± 5.4 (18)
Phenobarbital	52.4 ± 2.3 (9)	54.3 ± 10.7 (12)	54.3 ± 5.7 (9)	55.1 ± 6.8 (9)
Pentobarbital	54.6 ± 4.6 (15)	58.5 ± 6.3 (15)	57.5 ± 6.0 (15)	54.4 ± 6.1 (15)
Sulphaguanidine	3.5 ± 1.6 (12)		4.0 ± 1.3 (12)	
Promethazine	38.2 ± 6.1 (9)	38.3 ± 3.7 (9)	44.3 ± 5.0[1]) (9)	37.2 ± 5.1 (9)
Ethanol)	64.1 ± 7.5 (4)	53.2 ± 10.3[2]) (12)	53.5 ± 5.8[2]) (9)	48.6 ± 6.6[2]) (9)
Final blood ethanol[4])		0.7 ± 0.03 (6)	1.8 ± 0.1 (10)	3.8 ± 0.3 (6)

[1]) $0.02 < P < 0.05$ [2]) $P < 0.001$

[3]) In control experiments the ethanol concentration was 200 μg/ml

[4]) Arterial blood ethanol concentration (mg/ml) at the end of the experiments.

2. *Small intestine*

Perfusion of ethanolic solutions through the intestine

Absorption rates of the different drugs, when perfused through the small intestine in aquous or ethanolic solutions, are summarized in table 4. It can be seen, that ethanol is without effect on the absorption of some drugs with different physico-chemical properties. Only in one case was the absorption of promethazine just significantly enhanced. Final blood ethanol concentrations from some of the experiments are indicated at the bottom of table 4.

Table 5

Absorption from the rat small intestine at a steady blood ethanol concentration. The results are expressed as the mean ± standard deviation. Number of observations (i.e. 3 times the number of animals used) are indicated in brackets. The P-values are calculated by means of Student's t test.

Drug	Per cent absorbed within 10 minutes		Blood ethanol conc. mg/ml ± s	
	Control	Treated	Initial (n)	Final (n)
Barbital	48.1 ± 5.4 (30)	48.9 ± 4.4 (24)	0.9 ± 0.32 (8)	0.8 ± 0.19 (8)
		44.4 ± 5.9[1]) (24)	1.4 ± 0.08 (8)	1.3 ± 0.05 (8)
Promethazine	33.3 ± 8.1 (24)	33.6 ± 7.6 (24)	1.5 ± 0.08 (8)	1.4 ± 0.15 (8)

1) 0.02 < P < 0.05

Absorption during a steady blood ethanol concentration

During a period when the blood ethanol concentration was 1-1.5 mg/ml, experiments were performed with barbital and promethazine, the results of which are shown in table 5. As in the experiments shown in table 4, ethanol was without any clear cut effect on the absorption. In the experiments with the higher blood ethanol level, the absorption of barbital was decreased just significantly, which may have resulted from the combination.

pH and volume variations in the small intestine

In all experiments, regardless of the presence of ethanol in the intestine or in the blood, the final pH of the drug solutions was about 6.8. The volume correction factors were always about 0.9, indicating a slight absorption of water.

In vitro experiments

To exclude any influence on absorption by changes in blood supply to the absorbing area, some *in vitro* experiments were made on the absorption from isolated perfused segments of jejunum. In these experiments, treatment with ethanol was made in a way such as to imitate the *in vivo* experiments, i.e. 1.5 mg/ml or 1.0 and 2.0 % (w/v) of ethanol on the serosal

Table 6

Absorption from the rat small intestine *in vitro*

The amount of drug absorbed from the intestinal lumen into the bath, is expressed as μg per 100 mg intestine (dry weight) ± standard deviation per 30 min. Number of experiments are indicated in brackets.

	Treatment	μg/100 mg intestine/30 min [1]
Pentobarbital	N ethanol	34 6 ± 6.8 (8)
	1.5 mg ethanol/ml in the bath	36.2 ± 6.5 (7)
	2% (w/v) ethanol in the drug solution	35 7 ± 12.1 (8)
Sulphaguanidine	N ethanol	10.9 ± 6.4 (6)
	1% (w/v) ethanol in the drug solution	12.5 ± 3.5 (6)

[1] $P > 0.05$ I all cases.

and the mucosal side of the small intestine respectively It can be seen from table 6 that none of these treatments had any effect on the absorption of pentobarbital and sulphaguanidine

Discussion

In the present experiments the absorption from the stomach and the small intestine of some drugs with different physico-chemical properties, was studied under the influence of ethanol this was administered together with the drugs or was already present in the organism when absorption was being studied.

The positive effect of ethanol on the absorption of some of the drugs from the stomach, might be explained in three ways 1 A "solubilizing function" i.e. ethanol by transporting the drugs through the membrane, might enhance the absorption (TAPPEINER 1880) 2 Changes in the membrane characteristics due to ethanol. 3 An enhanced blood supply to the gastric mucosa under the influence of ethanol. The first suggestion can be ruled out, since a) ethanol had no effect on the absorption of pentobarbital and sulphaguanidine from isolated perfused segments of small intestine (table 6), as well as b) on the absorption of promethazine from the stomach (table 1 and 2) although this drug is fairly soluble in ethanol, even in its charged form, and further c) since it seems improbable, that concentrations of 5–10 % (w/v) in the stomach and 1.5 mg/ml in the blood would show a positive effect on the absorption of the same magnitude, as actually was found (table 1 and 2). Point a) and c) also apply to the

second possibility. A direct determination of the blood supply to the stomach and its mucosa being rather difficult in the rat, we checked the third hypothesis by means of the excretion of neutral red into the stomach after intravenous administration. This excretion proved to be about doubled under the influence of a steady state blood ethanol level of 1.5 mg/ml. It was further shown, that ethanol did not stimulate gastric acid secretion, since otherwise this might have resulted in an augmented excretion of the basic dye too. These results support the third hypothesis according to which the enhanced absorption of drug results from an increased blood supply to the gastric mucosa under the influence of ethanol.

According to this hypothesis, ethanol should only be capable of enhancing the absorption of drugs absorbed by passive non-ionic diffusion. This was actually the case, since only the absorption of the two barbiturates phenobarbital and pentobarbital, i.e. acids with pKa values of 7.2 (WADDELL & BUTLER 1957) and 8.1 (BRODIE *et al.* 1960) respectively was enhanced by ethanol whereas the base promethazine (pKa 8.6) was not absorbed neither with nor without ethanol in the stomach. In this connection it should be emphasized that ethanol, the absorption of which is independent of the pH, shows similar absorption pattern as the two barbiturates.

Although normally considered to stimulate gastric acid secretion, ethanol in high concentrations tends to inhibit acid secretion (RITCHIE 1965) and cause secretion of mucus, by irritation of the stomach mucosa, which results in an increase in intragastric pH (TRAVELL 1960). With concentrations of ethanol higher than 10% (w/v) in the stomach, the absorption is depressed again (table 1). From table 3 it can be seen, that with these concentrations the rise in pH during the experiment becomes very pronounced and furthermore marked congestion and often ulceration of the stomach was observed in these experiments, a phenomenon which was also observed in cats by TRAVELL (1960). In her experiments strychnine, administered into the ligated stomach was more fatal when given in a 25% ethanolic 0.15 N HCl solution, than when given in acid alone. She concluded, that an enhanced absorption had occurred, because of vasodilation, a rise in pH and a breakdown of the mucosa plasma barrier. According to these observations and to the pH partition hypothesis of SHORE *et al.* (1957) the rise in pH could be the reason for the depression of the pentobarbital absorption in experiments with 15 and 20% (w/v) ethanol in the stomach, since this would tend to cause ionization of pentobarbital. In the 16 experiments with 20% (w/v) of ethanol in the stomach, however, no correlation was found between the final pH and absorption in the individual experiments. From table 1 it

can further be seen, that ethanol depresses its own absorption like that of pentobarbital with high concentrations. This would not be the case if the high pH was responsible for the depression of pentobarbital absorption, since ethanol absorption is independent of pH It is therefore concluded that the depression of absorption in these experiments, is rather the consequence of the strong irritation of the stomach wall, possibly resulting in destruction with coagulation of the mucosa and serum as well as haemorrhagic exudations. Another important factor is the general and circulatory depression caused by the anaesthesia together with the high amounts of ethanol absorbed in these experiments. The rise in the pH is considered to be the result of an enhanced secretion of mucus together with the above mentioned exudations. This is also reflected in the volume correction factors (table 3), which rise in relation to the final pH although the dilution might to some extent be due to the osmotic effect of ethanol.

In the small intestine, ethanol had no clear cut effect on the absorption of the drugs tested (table 4 and 5) However the absorption rates for the drugs investigated were in good agreement with what could be expected, according to their degree of ionization and lipoid solubility of the uncharged form (BRODIE & HOGBEN 1957 VOGT 1965)

Absorption by passive non-ionic diffusion is considered proportional over a wide concentration range (SCHANKER *et al* 1958), which means that the percentage absorbed is constant. That absorption of ethanol in experiments with high concentrations of 0 5 1 0 and 2.0 ⁄ (w/v) is lower than in experiments with the very low concentration of 200 µg/ml is, how ever not surprising, since the experimental period is too short to allow an adequate absorption from these high concentrations.

The absorption capacity of the small intestine is very great (table 4 and 5) With regard to the absorption of phenobarbital and pentobarbital, it is conspicous that 2 to 3 times the percentage absorbed from the stomach in one hour is absorbed from the small intestine in about 10 minutes. The absence of effect of ethanol on the intestinal absorption of drugs is therefore considered to be the result of the very marked absorption area here thus providing optimal conditions, which could no longer be changed by relatively small variations in blood supply Because of these considera tions, it is not surprising, that although ethanol within certain limits is capable of enhancing the absorption from the stomach, no such effect was detected in the small intestine. That there is a positive correlation between blood supply and absorption from the small intestine, was clearly shown by WINNE (1966), using for instance 5-hydroxytryptamine and histamine for the production of changes in the blood supply to the small intestine.

The conclusion of the present study is, that although ethanol within

certain limits, is capable of significantly enhancing the absorption of some drugs from the stomach it is hardly likely that this effect will have any practical significance since no such effect was observed on the absorption from the small intestine from which the major part of drug absorption takes place. This conclusion is contrary to that of OTTO (1954) SPANN (1955) and MELVILLE *et al* (1966), but in agreement with that of GRAHAM (1960). It should be emphasized, that higher drug concentrations in the blood caused by the simultaneous ingestion of ethanol, might be explained by an effect of ethanol on drug distribution after gastrointestinal absorption (GREISER & SOEHRING 1967 SEIDEL 1967) or an interaction with drug metabolism (SCHÜPPEL 1967)

Summary

The absorption from the stomach and small intestine of some drugs with different physico-chemical properties was studied in rats, under the influence of ethanol. This was administered either together with the drugs, or was present in the organism in a steady concentration during the time of absorption Ethanol was found capable of significantly enhancing drug absorption from the stomach This, however only applied to drugs, which are absorbed fairly well even without ethanol because of their degree of ionization and lipoid solubility at the pH of the stomach. It was shown that this positive effect of ethanol was due to an enhanced blood supply to the gastric mucosa When administered in concentrations higher than 10 / (w/v), this positive effect of ethanol was again abolished, because of its irritating effect on the gastric mucosa. By the method used, ethanol was shown to be without any influence on the absorption from the small intestine. This is considered to be due to the presence of near optimal conditions for absorptions at this site

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Inhibition of Carbonic Anhydrase Activity of Whole Erythrocytes

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The difficulty of relating enzyme inhibition as measured *in vitro* on dilute enzyme solutions to the effects of various enzyme inhibitors on the whole cell is well known to pharmacologists. The physical and spatial state of the enzyme system in the cell and the effect of differences in transport of compounds to and from this system make it unlikely that there would be a close correlation

DAVENPORT (1946) was the first to review these problems with regard to carbonic anhydrase and pointed out the discrepancy between the *in vivo* and *in vitro* effects of certain inhibitors. At present some discrepancies can be satisfactorily explained by differences among the inhibitors with regard to their physiological distribution (WISTRAND *et al* 1961) and metabolic fate (MAREN 1956). Some, however still remain in doubt i.e., in the case of the thiazide type of inhibitors (BEYER & BAER 1962 MAREN & WILEY 1964).

The need of a method for measuring the effects of inhibitors on the carbonic anhydrase activity of whole cells therefore initiated the present study

The procedure to be described is an extension and a modification of a method originally proposed by KEILIN & MANN (1941). It makes use of the fact that methaemoglobin in erythrocytes acts as an indicator of the changes in intracellular pH. By suddenly exposing such cells containing methaemoglobin to carbonate solutions, the intracellular reaction $H_2CO_3 \leftrightarrow CO_2 + H_2O$ catalysed by carbonic anhydrase, is driven to the right. The rate of this reaction is reflected in the velocity of the shift from acid to alkaline methaemoglobin which can be measured spectrophotometrically The incubation of the cells with carbonic anhydrase inhibitors capable of permeating into the cells reduces the rate of this reaction The

present work describes the technique and how the activities of several inhibitors to the intracellular enzyme system of different types of erythrocytes are related to the known activities of the same inhibitors in dilute enzyme systems

Such a comparison may shed light on alterations in activity associated with disruption of morphological aggregates such as is known to occur for dehydrogenase (ZIEGLER & LIMMANE 1958) It will be shown however that whole cell inhibition correlates reasonably well with that predicted from a knowledge of the kinetic properties of the main carbonic anhydrase isoenzymes HCA *A* HCA *B* and HCA *C* (NYMAN 1961) contained in the *human erythrocyte*

Methods

Preparation f methaemoglobin corpuscles

The erythrocytes of heparinized animal blood or citrated human blood were separated from plasma by centrifugation and thereafter suspended (1 1) in saline (0 155 M NaCl in water). The suspension was gently shaken. After repeated centrifugation the cells were treated with 0.152 M (isotonic) sodium nitrite for 5 minutes so as to convert the whole of the intracellular haemoglobin into methaemoglobin. The sodium nitrite was removed by several washings with saline. The cells were finally suspended 1 120 in saline giving a cell count of about 10^4 cells/mm³ as measured in n electronic particle counter (Celloscope ® model 101).

To test whether this treatment had any effect on the carbonic anhydrase activity portions of rabbit cells were taken out after each step in the above procedure and analysed after lysis for their enzyme activity according to the method of PHILPOT & PHILPOT (1937). The activities were compared with those of the untreated cells from the same rabbit. The lysed methaemoglobin corpuscles contained 2160 enzyme units per ml (e.u./ml) as compared with 16.4 e /ml for the control cells. This higher activity was probably due to better packing of the washed cells after centrifugation. The rate of uptake of sulphonamides by these methaemoglobin cells was similar to that of untreated cells as seen in fig. 1 for CL

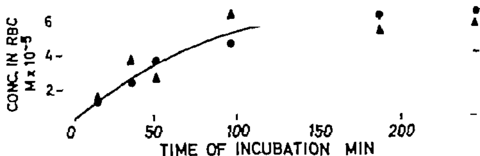


Fig. 1 Uptake of the highly ionized inhibitor CL 11366 at pH 7.0 by rabbit methaemoglobin corpuscles (● —— ●) and by control cells (▲ —— ▲) at +22 The cells were incubated with 2.8 × 10^{-3} M of the inhibitor

11366 (table 1) highly ionized inhibitor known to diffuse slowly across cell membranes (WISTRAND *et al* 1961). Similar results (not shown) were btained using a more rapidly permeating inhibitor acetazolamide This indicates that the washings and nitrite treatment had no great effect on enzyme activity o permeability f the erythrocytes.

Spectrophotometry of methaemoglobi corpuscles

Methaemoglobin behaves lik an indicator ver the pH-range 6.5 to 9.5 (AUSTIN & DRABKIN 1935). The bsorption spectrum of methaemoglobin in the cells is similar to that of the pigment in solution. This makes it possible to use the intracellular methaemoglobin for the spectrophotometric measurement of the pH in erythrocytes (DRABKIN & SINGER 1939) I the present study the problem was to perform spectrophotometry on suspensions of red cells where part of the extinction is caused by light scatter by the corpuscles. It was found, however that the extinction due to scattering is constant nd insensitive to the number of cells in the suspension, provided this number exceeds 3×10^4 cells/mm³ solution when using 1 cm cuvette. The number of cells in the suspension could vary between 3 to 15×10^4 cells/mm³ with ut affecting the results.

From bsorption curves taken between 400 and 700 mμ it was found that the bsorptivity ratio between suspensions f acid (pH 6.5) and alkaline (pH 9.9) methaemoglobi corpuscles was at maximum between 540 to 590 mμ In most of the studies the wave-length chosen was 560 mμ.

For the measurement of the rate f the shift from acid to alkaline methaemoglobin i the cells after addi g a carbonate solution to the acid cells, the increase in bsorbance during this hift was f llowed spectrophotometrically using double-beam instrument (Beckman, model DB) with thermostatically controlled compartment and connected to direct linear absorbance recorder (Sargent, model SRL). A single-beam spectrophotometer (Beckman, model B) with direct linear transmission recorder (Varian, model G 10) was used in some experiments.

The pH of the cell suspension was first adjusted to 6.5 by adding 0.1 ml of 0.1 M phosphat buffer (1 75 part of 0.1 M KH_2PO_4 + 1 00 part of 0.1 M K_2HPO_4) to 3 ml of the suspension. The cuvettes, each containing 3.1 ml f this suspension of acid methaemoglobin corpuscles, were brought into the sample compartment. The instrument was balanced at 100 / transmission. Before any significant settling of the corpuscles had occured, i.e. 2 3 minutes, 0.2 ml of 0.2 M Na_2CO_3 solution and 0.2 ml of saline were injected simultaneously into the test and reference cuvette, respectively Some air was also injected to insure rapid mixing. The addition of sodium carbonat brings the acid methaemoglobin into the fully alkaline form since the pH of the suspension increases to 9.8 at +22 At pH 9 4 more than 95 / of the methaemoglobin is in the alkaline form (AUSTIN & DRABKIN 1935). The increase in bsorbance due to this shift was followed on the recorder until the asymptotic value of the reaction was reached, usually at scale deflection f 0.5–0.6 optical density (OD) units. By titrating the acid cell suspension with Na_2CO_3 and following cha ges both in pH measured with glass electrode t 22 and in optical density it was f und that the 50 % lkaline methaemoglobin point t pH 8.12 (DRABKIN & SINGER 1939) was reached at the optical density of 0.25–0.30 pH 7 4 was reached at 0.1 OD-unit.

Typical tracings of experimental runs are seen i the figures 3 4 and 5. The asymptotic value of the curves was called 100 / alkaline methaemoglobin. The probable ionic events occuring after the addition of sodium carbonate are depicted in fig. 2. Bicarbonate and carbonate ions enter the cell through exchange with chloride, the divalent carbonate ion, however at much slower rate than the univalent bicarbonate ion (P R ST 1940). I combination with hydrogen ions carbon dioxide is formed which diffuses out. The rate of the observed reaction will depend on enzymic catalysis and on other factors in our system

Table I

Physical nd chemical properties f eight sulphonamides as related t their activities as carbo ic anhydrase inhibitors to the enzymes of intact or haemolysed erythrocytes.

1	2	3	4	5	6	7	8	9	10
Structure	Name or N	Mol. wt.	pKa_1 and (pKa_2)	Ether partition coeff	k M ?	Conc. for 50 reduction of cell activity µM		Relati e inhibi tory activity against	
						I ?	I	Haemo-lysate	Whole cells
	CL 11366	320	3.2 (9.0)	0 001	0.05	4	126	12.4	7 9
	Methazolamide	234	7.2	0.62	0.65	10	112	1 0	[illegible]
	Dichlorphenamide	305	8.3	11.0	2.0	16	100	0.3	2.0
	Ethoxzolamide	258	8.1	140	0.02	22	105	31.0	1.5

	Acetazolamide	222	7.4 (9.1)	0.14	0.62	32	76	(1.0)	(1.0)
	Benzthiazide	432			0.63	80	116	1.0	0.4
	Sulfanilamide	172	10.4	0.15	57	1150	2080	0.01	0.03
	Chlorothiazide	296	6.7	0.04	10	–	–	0.06	–

Column 2 Names according to New Drugs, 1965. Column 3 4 5 From WISTRAND *et al.* (1961). Column 6 From dog haemolysate t 37 MAREN (1963a) Column 7 8 From figure 7 A D Column 9 From values of column 6 (acetazolamide = 1.0). Column 10 From values of column 7 (acetazolamide = 1.0).

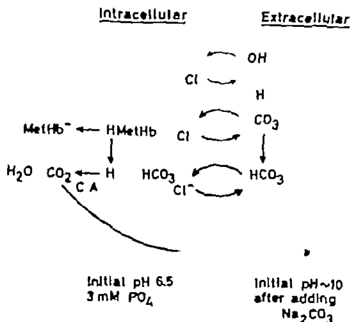


Fig. 2. Probable ionic events after the sudden addition of 12 mM of sodium carbonate to methaemoglobin corpuscles suspended in a 3 mM phosphate buffer of pH 6.5.

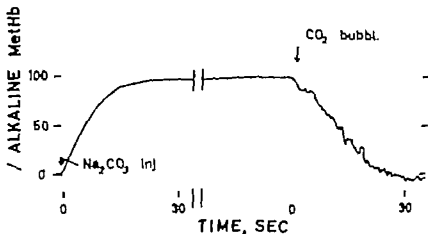


Fig. 3 A typical tracing of the reversal of the shift from acid to alkaline methaemoglobin within rabbit erythrocytes after the sudden exposure of the cells to CO_2.

where the reaction has proceeded f r about 1 second before being recorded. Such possible factors are amount of substrate and rate of removal of product. The substrate concen tration depends on the supply of chloride and the rate of interchange f this ion with bicarbonate (DIRKEN & MOOK 1931) and is also dependent on the rapid supply of hydrogen ions by buffers inside the cell. The latter are made vailable *in vivo* mainly from oxygenatio of haemoglobin and it is, therefore, uncertain what the supply would be in cells containing methaemoglobin. It is difficult here to evaluate the relative role of these factors. The capacity of the detection and recording system (0.5 second response f full scale deflection) was not rate limiting (BIAS *et al.* 1959). The indicator change per se is complete in 0 002 seconds or less (ROUGHTON & RUPP 1958) Enzyme catalysis is not rate limiting in our system or *in vivo* before over 99 / of the enzyme activity is bolished as will be discussed below

The shift from the acid to the alkaline form f methaemoglobin is reversible. This is illustrated in fig. 3. When carbon dioxide (95 % CO_2 + 5 / O_2) was bubbled through suspensions where the dehydration reaction had gone to completion, the methaemoglobin was fully reversed into its acid form. Studies f the hydration reaction and the effect of inhibitors on this are the subject of separate study

E idence for the intracellular site of the observed reaction

To see whether the reactions recorded occur inside the cells and not in the extracell lar fluid due to the presence in this fluid of methaemoglobin and enzyme from lysed cells, the following experiments were done.

The suspension of acid methaemoglobin corpuscles was centrif ged. The supernatant fraction was clear and almost colourless and there was no detectable shift in absorbance t 560 mμ after the ddition of sodium carbonate. The amount of enzyme and methaemoglobin in the supernatant fraction was analysed and compared with that of the packed cells. The degree of haemolysis could then be estimated. The highest figure btained from measurements of enzyme activity was 1.4 Y haemolysis. Methaemoglobin was measured spectrophotometrically in the supernatant at pH 6.4 using one of the absorption maxima f acid methaemoglobin, 405 mμ. The concentration of methaemoglobin in the extracellular fluid corresponded to lysis of 1 1 % of the total umber of cells

I the same way it was found that adding sodium carbonate to the acid suspension like in the experimental runs did not induce haemolysis before standing t room temperature for 2 3 hours.

From the above it is concluded that the observed methaemoglobin reactio occurs inside the erythrocytes. A further indirect evidence is the fact that the reduction f the reaction rates is related to the amount of inhibitor inside the cells.

Effects f carbonic anhydrase inhibition

When a carbonic anhydrase inhibitor such acetazolamid (table 1) was added to the suspension and llowed to equilibrate fully with the cells the reaction rates became slower with increasing inhibitor concentrations (fig 4). The same asymptotic alkaline methaemoglobin alue as seen for control cells was reached provided the addition of the inhibitor had not changed the pH of the cell suspension. Acetazolamide, 10^{-3} M did not affect the b- sorption curves of the acid or alkaline cells. Above certain concentrations, different for various inhibitors, no further depression of the reaction rate could be achieved even after prolonged incubation times. It was therefore assumed that the enzyme activity of these cells was completely nhibited. The activity of these inhibited cells was rather similar to that of cells from neonatal infants which contained small amounts of carbonic anhydrase (see results section and fig 5). Incubati g the cells for 3 hours at +22 ith 10− M of the

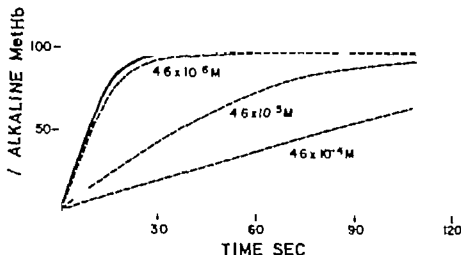


Fig. 4. Lack of effect of the control sulphonamide CL 13850, 10^{-3} M, (unbroken line) in comparison with the graded effects of increasing concentrations of acetazolamide (dashed lines) on human erythrocytes.

control sulphonamide CL 13850 a N³-t-butyl derivative of acetazolamide (MAREN 1956) devoid of inhibitory activity had no effect (fig. 4). It has pKa of 7.3 and should diffuse into the red cells at a rate comparable to that of acetazolamide.

The difference in reaction rates between the maximally inhibited and the non-inhibited cells reflects the catalytic activity of carbonic anhydrase. Any reaction rate in between represents an inhibitory effect on the enzyme.

The initial part of the reaction curves wer straight and the rates, up to pH 7 4, were expressed as a change in optical density per unit time, Δ OD 560 mμ/second. The enzymic activity observed was taken to be the rate of the measured reaction minus that of the maximally inhibited cells.

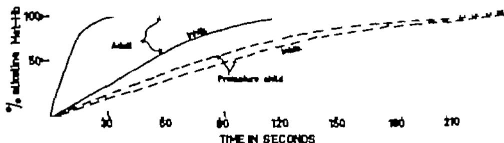


Fig. 5 Tracings from erythrocytes of an adult (———) and of a neonatal infant (- - - -). The lower curves (Inhib.) of each pair were obtained after the cells had been incubated with 1.2×10^{-3} M of acetazolamide.

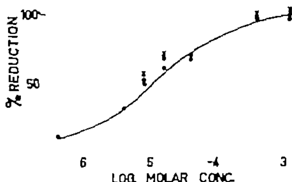


Fig. 6. The effects of methazolamide on human cells t +4 (x) +22 (O), and t +37 (●). The line was drawn through the values at +37

If the effects at equilibrium of the inhibitors were expressed as per cent reduction f the observed enzyme activity and plotted gainst the concentrations, the curves shown in fig. 7 were obtained.

Effects of temperature

At +4 and +22 the reaction velocities were 15 and 50 / respectively of those at 37. The change in temperature did not affect the bsorptivity ratio between acid and alkaline methaemoglobin corpuscles.

Inhibition studies were also done using the relatively lipid soluble inhibitor methazolamide (table 1) which equilibrated with the cells i 60 minutes even t +4. No significant effect on the inhibitory activity of this substance was seen with changes in temperature (fig. 6).

Reproducibility of the method

Washed and nitrite-treated cells suspended in six volumes of saline could be stored t +4 for 2 3 days without loss of activity. The standard deviation of single determinatio of activity was found to be +4.8 as calculated from 12 control runs from one day. The corresponding figure from 8 runs on maximally inhibited cells was +6 %.

Results

Incubation times necessary for equilibrium

From studies *in vivo* (WISTRAND *et al.* 1961) and on erythrocytes (HOLDER & HAYES 1965) or kidney tubular cells *in vitro* (BEYER & BAER 1962) it is known that carbonic anhydrase inhibitors vary considerably in their ability to penetrate into and accumulate in different types of cells.

Moreover some inhibitors seem to vary in their need for time of contact with the enzyme to ensure maximal inhibition, as also found in dilute enzyme systems (WISTRAND *et al* 1961 MAREN 1963a).

The whole cell method allows the study of the net effect of these factors, since the time of incubation necessary to reach inhibition equilibrium reflects the time it takes for the inhibitor to permeate into the intracellular (GRAY *et al* 1961) enzyme and react with it. For these studies the cells were incubated with the inhibitor for various periods and then analysed for their catalytic activity and content of inhibitor The inhibitors were determined according to the method of MAREN *et al* (1954b), except for chlorothiazide and sulphanilamide which were analysed by the method of BRATTON & MARSHALL (1939)

The uptake of the highly ionized (table 1) inhibitor CL 11366 by normal and nitrite treated cells at 22 is seen in fig. 1 at 37° the uptake and inhibition were already maximal after 20 minutes. A large concentration, > 10⁻³ M allows such a rapid uptake of the inhibitor by the cells, that maximal inhibition is reached before an equilibrium between extra- and intracellular inhibitor concentration has been achieved.

Less ionized (at pH 6 4) and more lipid soluble inhibitors, ethoxzolamide and acetazolamide, in that order were taken up more rapidly by the cells and the maximal inhibition after incubation was reached earlier than in the case of CL 11366 Thus after incubation with ethoxzolamide the uptake and the ihibition of the cells had reached its maximum before five to ten minutes at 22°

To ensure equilibrium an incubation time of 60 minutes was sufficient at +37° even for CL 11366 the most poorly penetrating of these inhibitors. At +4 however several hours of incubation were needed for CL 11366 whereas 60 minutes were enough for the more lipid soluble methazolamide.

These findings are consistent with the view (WISTRAND *et al.* 1961 HOLDER & HAYES 1965) that the ionization and lipid solubilities of these compounds govern the speed by which they enter the red cells. Active transport by the mechanism of the kidney for dealing with acids also determines the concentration of some inhibitors in the tubular cells, as shown for CL 11366 and chlorothiazide (TRAVIS *et al.* 1966)

Effects of various inhibitors at equilibrium

The erythrocytes were incubated at 37° with increasing concentrations of inhibitors with different physical and chemical characteristics (see table 1) until inhibition equilibrium had been reached. The catalytic

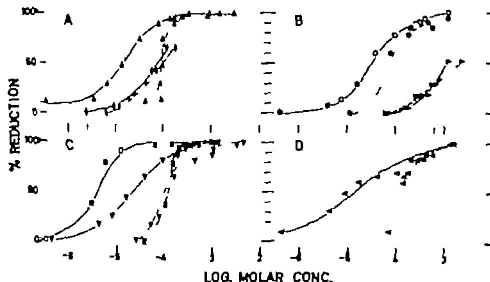


Fig 7 A–D Reduction (in %) of the catalysed reaction rate of human erythrocytes at various concentrations of inhibitors in the extracellular fluid, I_{free} (open markings) or within the cells, I_o (filled markings).

A ▲ and △ – dichlorphenamide, ⧫ and ◊ – benzthiazide
B ● and ○ – acetazolamide, ▶ and ▷ – sulfanilamide
C ■ and □ – CL 11366, ▼ and ▽ – ethoxzolamide
D ◀ and ◁ – methazolamide

activities of the cells were then measured at +37° as well as their intracellular content of inhibitor

The inhibitors were dissolved in water or when small concentrations (<1 %) were used in 0.9 % saline, by adding from 1–2 moles of NaOH to each mole of sulphonamide.

The results are illustrated in table 1 and fig 7 (A–D). In fig. 7 (A–D) the total intracellular concentration (I_o) and the concentration of inhibitor in the extracellular fluid (I_{free}) have been plotted against the degree of reduction of cell activity The same degree of maximal reduction of the cell activity is reached by the inhibitors with low K_I values ($<2 \times 10^{-7}$ M table 1) although different concentrations for I_{free} are needed for this, i.e. about 100 μM for the most active CL 11366 and from 500 to 1000 μM for the others within this active group These extracellular concentrations generated about 630 to 1000 μM of total inhibitor concentration, I_o, inside the cells at maximal reduction.

This maximal effect was not seen for the inhibitors with a $K_I > 10^{-6}$ M

which could not be dissolved in sufficiently high concentrations without upsetting the pH of the reaction fluid

The slopes of the response curves of the various inhibitors were parallel, with the exception of methazolamide (fig. 7D) the curve of which was for some unknown reason flatter than the others.

The concentrations of I_{free} and I_a necessary for a certain degree of reduction of cell activity is obtained for each inhibitor from fig. 7 A-D These measured concentrations agree reasonably well with those deduced from the knowledge of the kinetics of the purified human erythrocyte enzymes (conf Discussion).

The relative activities among the inhibitors calculated from the values of I_{free} at 50 / reduction have been compared with those calculated from the K_I-values at 37° of the same inhibitors reported by MAREN (1963a) from measurements on haemolysates of dog erythrocytes (table 1). The dog erythrocyte enzyme has a similar sensitivity to inhibitors as the human HCA *C*-enzyme. The latter enzyme is in turn more sensitive to inhibition than the other human enzyme, HCA *B* but the relative order of activity among the inhibitors of these isoenzymes is about the same (WISTRAND 1965) Approximately the same relationship between the inhibitors was seen against these whole human cells as against each separate isoenzyme.

CL 11366 with its low pKa of 3.2 (table 1) and therefore almost totally ionized when used in this technique was the most potent inhibitor However ethoxzolamide and dichlorphenamide, which were mostly un ionized under these test conditions were also very active. This would indicate that the state of ionization of the sulphonamides does not seem to be of major significance for their inhibitory activities in the physiological pH range, but only in their ability to penetrate into the cells. Sulphanilamide was the least potent substance and benzthiazide lay in between the extremes. Another thiazide derivative, chlorothiazide, showed no inhibitory effect when tested in concentrations up to 1 7 × 10^{-4} M

Reversibility of the inhibition

The inhibition of dog haemolyzate activity by acetazolamide is reversible (MAREN *et al* 1954a) The question was investigated whether this was also true for whole rabbit and human methaemoglobin corpuscles. The cells were saturated with inhibitor by incubating them for 2 hours at 37 with 4.5 × 10^{-4} M of acetazolamide, giving a total intracellular concentration, I_a, of 4 4 × 10^{-4} M in the human cells see fig. 8 Portions of the cells were then washed by suspending them (2 ml) in 50 ml of saline, gently shaking the suspension for about 10 minutes and again removing the cells by centrifugation. The cells were washed in this manner from 2

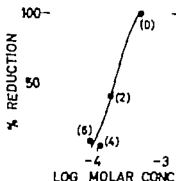


Fig. 8 Reversibility of the inhibition by acetazolamide. Erythrocytes, saturated with inhibitor were washed up to six times in saline. Abscissa concentration of inhibitor within the cells after each wash. Ordinate reduction of reaction rate in relation to those of control cells (= 0 %) and non-washed inhibited cells (= 100 %). Numbers within brackets are number of washes.

to 6 times. After each wash they were analysed for their content of inhibitor I_e, and catalytic activity

From fig. 8 it can be seen how repeated washing reduced the I_e at the same time as it restored the enzyme activity After four and six washes the incubated cells still contained 106 and 76 µM of acetazolamide respectively and the cell activity was then reduced to 5–8 % This corresponds well with the relation between I_e and degree of reduction of activity observed in the regular experiments shown in fig. 7B and also shows that the inhibition is reversible.

Activity of different types of erythrocytes

Erythrocytes of man, horse, rabbit, and mouse gave rather similar reaction curves before and after incubation with inhibitors but no systematic comparison was done with regard to catalytic activities or sensitivity to inhibition. As seen in fig. 9 it would seem however as if rabbit cells (one animal) are more sensitive to acetazolamide than human cells, although the cells were tested at 22 and 37° respectively which makes a close comparison difficult. The K_i of acetazolamide against the haemolysates of various species does not vary much (WISTRAND & RAO 1968) but differences should be more obvious at the high degrees of enzyme inhibition studied here, if certain cells contained a mixture of isoenzymes one of which was relatively insensitive to the inhibitor It has been shown by HANSSON (1965) that each single erythrocyte of the adult human contains both isoenzymes, HCA *B* and HCA *C*

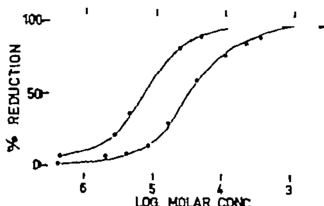


Fig. 9 Comparison of the effects of acetazolamide on human, (●) and rabbit (○) erythrocytes, analyzed at +37 and +22 respectively

Corpuscles from different adult individuals of the same species varied in their catalytic activities (for such data on lysed cells see LARIMER & SCHMIDT NIELSEN 1960), but differences were not large. No real effort was made to map out the variability in this respect. It should be noted that differences observed could be related to rate limiting factors other than the enzyme, as discussed in the Methods section

It was also of interest to study the red cells of premature children which are known to contain a low carbonic anhydrase activity (STEVENSON 1943). The blood of two such children, born 6 and 8 weeks, respectively before expected parturition were used one portion of each blood was taken for analysis of its enzyme activity after haemolysis and the other portion was analysed using the present whole cell technique.

The enzyme activities of the lysed cells were 32 and 37 e.u./ml which should be compared with the value of 1880 e.u./ml as found in one adult man. The reaction curve of the whole cells of one of the children compared to that of the adult is seen in fig. 5 The rate of the reaction of the child's cells was about 8 times slower but the same shift from acid to alkaline methaemoglobin was observed. Although technically difficult, an effort was made to study the sensitivity of the red cells of one of these children to acetazolamide Fig. 10 shows the inhibition curve compared to that of cells from the adult. The child's cells were more sensitive to inhibition

During the life span of erythrocytes several of their enzymes undergo some modifications (MARKS *et al* 1958) The question whether this also applies to carbonic anhydrase was tested.

The erythrocytes of an adult man were separated according to age, using the ultracentrifugal fractionation technique of GARBY & HJELM

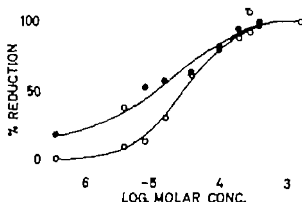


Fig. 10. Difference in sensitivity between erythrocytes of one adult and one neonatal infant (upper curve) to acetazolamide inhibition.

(1963). The centrifuged sample was divided into three layers. The top layer contained predominantly young cells, 0–20 days of age, whereas the middle and bottom layer contained the older cells. The cells of the different layers were analysed for their enzyme activity using both the whole cell technique and also after lysis. No certain difference in activity between the cells of the different layers was noted with either technique.

Incubating the cells with acetazolamide and measuring the degree of cell inhibition after this by using the whole cell technique, did not reveal any difference in sensitivity to the inhibitor between the cells of different layers.

Old and young erythrocytes of human adults therefore appear to behave kinetically in a similar way with regard to carbonic anhydrase activity

Discussion

If it is true that the enzyme activity is maximally inhibited in the cells incubated with $>10^{-3}$ M of the most active inhibitors, then carbonic anhydrase is responsible only for the 10–15-fold magnification of the rate seen in our system KERNOHAN *et al.* (1963) have estimated from measurements on concentrated human enzyme solutions, containing all isoenzymes but free of haemoglobin, that the enzyme in the red cell should be capable of multiplying the dehydration rate by 13000-fold and deduced that the rate of multiplication *in vivo* is dependent on enzyme concentration rather than substrate or product concentrations. Clearly therefore, rate limiting factors other than the enzyme operate in our system. Some of

these are briefly discussed in the Methods section. It should be added here that the net effect on the enzyme activity of the ions present in our system cannot be foreseen The carbonate ion has been claimed to be either inhibitory to bovine enzyme (ROUGHTON & BOOTH 1946) or has no effect (MAREN 1963a) on the activity of dog haemolysate the phosphate ion was found by DE VOE & KISTIAKOWSKY (1961) to act formally as an activator and competitive inhibitor to bovine enzyme but had no effect, when tested by GIBBONS & EDSALL (1964) to human HCA *B* and HCA *C* chloride is inhibitory to both the human (DEVOE & KISTIAKOWSKY 1960) and bovine (ROUGHTON & BOOTH 1946) erythrocyte enzymes.

Moreover when the cell activity is measured under more physiological conditions as done by CONSTANTINE *et al* (1965) some unknown process other than enzyme catalysis is rate limiting. These investigators used a continuous-flow rapid reaction apparatus where small pH-changes in a *total* red blood cell suspension were measured during hydration-dehydration reactions.

In view of this there is a need for further analysis of the kinetic behaviour of the whole cell.

Assuming, however that the enzyme of our erythrocytes also had a capacity of a 13000-fold multiplication of the rate, the 10-15 fold acceleration of the rate seen here could be achieved even if only 0.077 or 0 115 / of the enzyme was active and thus ~*99.9* / inhibited. The *50* / reduction of the catalysed rate in our system would therefore not be reached until the total enzyme activity was inhibited by 99 95 /. ROUGHTON (1954) estimated that the rate of turnover of CO_2 into bicarbonate needs to be magnified 700-fold for an effective CO_2-evolution during the transit of blood through the lung capillaries at work. Hence to achieve this, only 5 4 / of the total enzyme activity is necessary An even larger excess of enzyme in relation to the physiological need is apparent in other organs i e the pancreas (RAWLS *et al* 1961) and kidney (MAREN 1963b) where the normal cell activity (output of HCO_3 from CO_2) is only 2 to 4 times larger than that at maximal enzyme inhibition. Due to the several thousand fold excess of enzyme in these organs, impairment of physiological activity is not seen before at least 99 / of the enzyme has been inhibited.

The relation between enzyme inhibition and physiological effects first outlined by DAVENPORT (1945) has been extensively studied and clarified by MAREN (1963b) in the case of animal tissues, assuming the presence of *one* enzyme in these tissues.

However matters are complicated in human tissues i.e. red cell kidney and lens (WISTRAND & RAO 1968) which contain two possibly three, carbonic anhydrases of which the erythrocyte isoenzymes HCA *B* and HCA C have been studied in detail and found to differ kinetically as

Table 2

Kinetic parameters of the human erythrocyte carbonic anhydrases, HCA *C* and HCA *B*, of relevance for whole cell inhibition

Parameter	HCA *C* $HCO_3^- \rightarrow CO_2$	HCA *C* $CO_2 \rightarrow HCO_3^-$	HCA *B* $HCO_3^- \rightarrow CO_2$	HCA *B* $CO_2 \rightarrow HCO_3^-$	Reference
$\frac{V}{E_0}$ [1]) × sec. × 10^{-3} pH 7.05, +25	365	620	23	15	GIBBONS & EDSALL (1964)
K_m × mM pH 7.05 +25	68	14	32	2.6	GIBBONS & EDSALL (1964)
Acetazolamide, K × µM at +0.5		0.02		0.22	WISTRAND (1965)
Acetazolamide, K × µM at +10		0.036		0.51	WISTRAND (1965)
Acetazolamide, K × µM at +37°		0.1		1.0	extrapolated[5])
Intracellular conc., E_0, µM	18		81		NYMAN[2]) (1961)
	20		136		H H NIELSON[3]) person. comm.
		150			MAREN *et al* [4]) (1961)

[1]) Temperature coefficient for dehydration reaction = 1 40/10 and for hydration reaction = 1.45/10 for human erythrocyte enzyme (KERNOHAN *et al.* 1963)

[2]) Calculated from separation procedure to be 18 and 81 µM for HCA *C* and HCA *B* respectively

[3]) Immunological titration.

[4]) From binding data using potent inhibitors.

[5]) For dog haemolysate K was 0 062 and 0.014 µM at 37 and 0 respectively and did not change over the pH-range of 7.8–6.4 (MAREN 1963).

summarized in table 2. By use of these kinetic parameters and the Michaelis relation

$$V_E = \frac{V_{max} \times S}{K_m + S} \quad (1)$$

where V_E is the catalysed reaction velocity and S is the substrate concentration *in vivo* it can be calculated that HCA *C* accounts for about 60 % and the other isoenzymes HCA *B* and HCA *A* for 40 % together of the total enzymic activity. From the relations

$$K_I = \frac{(E_0 - EI) \times I_{free}}{EI} \quad (2)$$

assuming a non-competitive type of inhibition (LEIBMAN *et al.* 1961) and

$$i = \frac{EI}{E_0}$$

where K_I is the enzyme inhibitor dissociation constant at 37°, E_0 is molar concentration of enzyme (E_{01} is HCA *C* and E_{02} is HCA *B* + HCA *A*) and EI is inhibitor bound enzyme, the fractional enzyme inhibition (i or i_1 for HCA *C* and i_2 for HCA *B*) can be obtained from

$$i = \frac{I_{free}}{I_{free} + K_I} \quad (3)$$

Now if total activity of the enzyme mixture is called 100 %, of which HCA *C* accounts for a % and the HCA *A* and HCA *B*-enzymes for (100 − a) % the per cent inhibition of total enzyme activity will be

$$i = i_1 \times a + i_2 (100 - a) \quad (4)$$

or

$$i = \frac{I_{free}}{I_{free} + K_{I1}} \times a + \frac{I_{free}}{I_{free} + K_{I2}} \times (100 - a) \quad (5)$$

K_{I1} and K_{I2} are inhibitor constants for HCA *C* and HCA *B* and HCA *A* respectively. Using the values of K_I for acetazolamide at +37° (table 2), and assuming values for a between 0 and 100 %, the relation between total enzyme inhibition and free inhibitor concentration for this inhibitor is obtained from equation (4) and is illustrated for i-values above 95 % in fig. 11. The inhibition curves of enzyme mixtures will be flatter than those of single enzymes. The presence of an enzyme of the B-type with a relatively high K_I-value, will be apparent as a need for high inhibitor concentration for the high degrees of inhibition. This might explain why DAVENPORT (1945) found sulphanilamide to be less active against intact erythrocytes

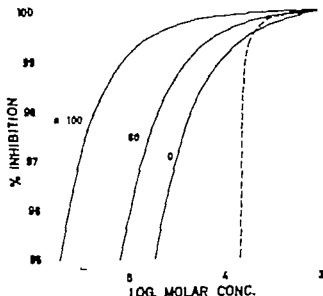


Fig. 11 Theoretical curves (from equation 5) relating the concentrations of freely diffusible cetazolamide, I_{free}, to the inhibition of total enzymic activity in human erythrocytes at +37° a = fractional activity (in %) due to HCA *C*. Broken line is intracellular concentration I_{in} (from equation 6) at the concentrations of I_{free} which give the inhibition curve if a = 60.

than predicted from experiments on haemolysates where 50 % inhibition values are obtained.

The newborn's blood with its low enzymic activity (see Results section) contains relatively small amounts of HCA *B* as indicated from immunological data (WISTRAND & RAO 1968). These cells should therefore be more sensitive towards inhibitors than those of the adult, particularly at high degrees of inhibition as indeed was found to be the case, fig. 10. The more sensitive rabbit blood (fig. 9) would also indicate a lack or reduced titer of an enzyme of the B-type in this blood.

The intracellular concentration, I_{in}, at given value of I_{free} can be obtained from the relation

$$I_{in} = E_1I + E_2I + 0.63 \times I_{free} \qquad (6)$$

where E_1I and E_2I equals the amount of inhibitor bound to HCA *C* and HCA *B* + HCA *A* respectively or

$$E_1I = i_1E_{o1} \quad \text{and} \quad E_2I = i_2E_{o2}$$

0.63 is the volume of cell water (WINTROBE 1946).

The values for I_o have been plotted against the inhibition values seen when HCA C accounts for 60 % of the activity thus the values of I_{free} giving a certain degree of total enzyme inhibition at a = 60 will generate the I values of fig. 11 It is seen that when 95 % of the enzyme activity is inhibited, i.e. when a physiological effect should start to appear according to ROUGHTON (1954) the I_{free} for acetazolamide should be 8.3 μM giving an intracellular concentration, I_o, of 146 μM (33 μg/ml)

Such a concentration of freely diffusible acetazolamide in human plasma is obtained at a total plasma concentration of ~100 μM (or 22 μg/ml) assuming 92 % plasma binding (TRAVIS *et al* 1966).

Data which relate partial respiratory effects to inhibitor concentrations *in vivo* are scarce. However POCIDALO *et al* (1959) found a widening of the arterial-alveolar PCO_2-gradient in man at 75 minutes after 12.5 mg/kg orally of acetazolamide, at a time when the plasma concentration of I_{free} should be 12 μM (LEHMANN *et al* 1968).

When the rate of the catalysed reaction of our cells was reduced by 50 %, the I_{free} for acetazolamide was 32 μM (table 1) which corresponds to 98.58 % inhibition according to fig. 11 However the degree of enzyme inhibition should be even higher or 99.95 % as deduced from the relation between observed and calculated capacity for enzymic acceleration. This discrepancy could be explained either by the inhibitor being more active than assumed in table 2, or by a smaller excess of enzyme activity than that assumed At this degree of inhibition the most active inhibitors gave an intracellular concentration, I_o, of about 100 μM which is somewhat less than those calculated. This could be due to a smaller molar amount of enzymes in the cells than that reported (table 2).

Our results support the view of an enzyme inhibition of >99 99 % at the highest inhibitor concentrations ($>10^{-3}$ M) used and are in contrast to those reported by CONSTANTINE *et al.* (1965) who found that a 254 to 590-fold acceleration of the CO_2-uptake of human erythrocytes remains in spite of the fact that the cells had been incubated for 4 hours at 37° with $1\ 13 \times 10^{-3}$ M of acetazolamide. This corresponds to only between 72 and 96 % enzyme inhibition in their system. The reason for these low inhibition values is not clear

Only a few data on the effect of inhibitors on whole erythrocytes using other techniques are available. Using a manometric technique for recording the uptake or delivery of CO_2 from a suspension of dog erythrocytes, DAVENPORT (1945) found thiopene 2-sulphonamide ($K_I = 1\ 6 \times 10^{-7}$ M at 0° and +10°) to be maximally inhibitory at a concentration of $6\ 2 \times 10^{-4} - 1\ 5 \times 10^{-3}$ M, whereas $> 2.9 \times 10^{-3}$ M of sulphanilamide had to be used for the same effect. JACOBS & STEWART (1941) using an osmotic method where the change in volume of the red cell caused by the

osmotic effect of the CO_2-reaction within the cell is used as a criterion for catalysis, found sulphanilamide in concentrations of 2×10^{-6} M to be inhibitory

Our results and those cited above indicate that carbonic anhydrases when located intracellularly do roughly behave to inhibitors, as can be predicted from the data obtained *in vitro* on dilute enzymes.

Summary

A method is described which allows the study of the effect of inhibitors on the carbonic anhydrase activity within whole erythrocytes. It is essentially a changing-pH method where the speed of the intracellular pH shift, due to the catalysed dehydration of carbonic acid within the cells is followed spectrophotometrically using methaemoglobin as an internal indicator

Eight sulphonamides of varying physical properties and potency were studied against human erythrocytes at +37° The order and range of relative activities among the inhibitors were similar to that seen for the erythrocyte haemolysates, CL 11366 being the most and chlorothiazide the least potent.

A similar maximal inhibitory effect was achieved only with inhibitors with a K_i of <1 µM. The maximally achieved reduction of the catalysed reaction should correspond to >99.99 / inhibition of the total enzyme activity if account is taken of the presence in human erythrocytes of two isoenzymes of carbonic anhydrase showing different kinetic behaviour Among the potent inhibitors were those almost totally ionized or un ionized under the condition of test. Ionization only appeared to affect the rate of permeation into the cells. The inhibition was reversible. The erythrocytes of the neonatal child had a very low catalytic activity which appeared more sensitive to inhibitors than that of the cells of the adult human.

Erythrocytes of different ages from the same adult behaved in the same way kinetically

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Division of the American Cyanamid Co., dichlorphenamide and chlorothiazide by Merck Sharp & Dohme, benzthiazide by Chas. Pfizer & Co and ethoxzolamide by The Upjohn Co USA.

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Acute Hepatocellular Damage Caused by Oleoresin of the Male Fern in the Rat An Electron Microscope Study

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(Received August 25 1967)

Extracts prepared from *dryopteris filix-mas* exert a toxic effect on the liver of rabbits and rats, when tested by some liver function tests (NOSSLIN & MORGAN 1965). It has been shown that the mechanism of this action involves a decreased uptake phase of dyes by the liver cells (SHERLOCK 1964). The later experiments in man (TAKKI 1967) showed a decreased excretion into the bile and hepatocellular damage It therefore seemed of interest to examine the possible changes in the fine structure of the hepatic cells after administration of *extractum filicis*

Material and Methods

White male Sprague-Dawley rats, weighing 200–250 g were used. The animals were fasted for 4 hr before the experiments. The rats were given orally 500, 200 and 50 mg/kg respectively of *extractum filicis* (Ph. F VII), calculated as crude filicin dissolved in 1% sodium cholate solution. A dose of 500 mg/kg corresponds to about the LD50 of the extract (AIRAKSINEN *et al.* 1967 TAKKI 1967). Each group consisted of eight rats except the group receiving 200 mg/kg, which consisted of 17 rats. The control group was given 1% sodium cholate solution.

Most of the rats were decapitated and exsanguinated 2 h after administration of the extract. However nine rats in the group of animals receiving 200 mg/kg were killed in groups of three animals 1 3 and 7 days after administration of the drug. Specimens of the liver were immediately fixed in 3% glutaraldehyde (SABATINI *et al.* 1963) in phosphate buffer (pH 7.2) for 1 hr at 4 and then put into phosphatesucrose (0.2 M sucrose in phosphate buffer pH 7.2). The samples were then fixed for 1 hr in veronal buffered (pH 7.2) 1 osmium tetroxide. After rinsing in distilled water the specimens were dehydrated in ascending series of ethyl alcohol and embedded in epoxy resin Epon 812 (LUFT 1961) Polymerization to suitable degree of hardness was effected by placing the specimen in an oven at +45 for about 48 hr Thin sections were cut with the Porter Blum MT 2 ultra-

microtome using glass knives, post-stained with lead citrate solution (REYNOLDS 1963) and examined with the Siemens Elmiskop I. Electron micrographs were taken at original magnifications of 3,000 to 16,000 and enlarged as desired.

Results

Control specimens showed the characteristic features of the normal hepatic cells (compare fig. 5). In the nucleus condensed chromatin was distributed in a thin layer around the periphery. Mitochondria with thin folds of the inner membrane projecting into the cavity of the mitochondrion were seen. There were two types of endoplasmic reticulum, the granular or rough-surfaced form and the agranular or smooth-surfaced form. Lysosomes and microbodies were also identified. Glycogen was abundant and tended to be concentrated in large masses.

Examination of liver specimens from animals treated with 500 mg/kg of filix extract revealed striking changes (table 1, fig. 1). The relative proportions of the granular and agranular endoplasmic reticulum had changed in such a manner that the former was reduced and the latter was increased. Cisternae of the granular endoplasmic reticulum were greatly distended and the ribosomes had become rare. Multiple vacuoles appeared in the cytoplasm, and there was distinct hypertrophy of the agranu-



Fig. 1. Rat liver two hours after administration of 500 mg/kg of Extractum filicis (calculated as crude filicin). General view showing four adjacent parenchyma cells. × 5,700.

Table I

The effect of different doses of Extractum filicis on the fine structure of the rat liver cell.

Dose mg/kg (number of animals)	Time until sacrifice	Endoplasmic reticulum		Glycogen	Cytoplasm	Mito-chondri	Nucleus, cell membrane, bile canaliculi, space of Disse
		smooth	rough				
Controls. (10)	–	N	N	N	N	N	N
500 mg (8)	2 hrs.	increased +++	decreased +++	decreased +++	vacuolizatio ++	changes +	N
200 mg. (8)	2 hrs.	increased +++	decreased +++	decreased +++	acuolization ++	N	N
200 mg. (3)	1 day	increased +++	decreased +++	decreased +	vacuolization +	N	N
200 mg. (3)	3 days	increased +	decreased +	N	N	N	N
200 mg. (3)	7 days	N	N	N	N	N	N
50 mg. (8)	2 hrs.	increased ++	decreased ++	decreased +(?)	vacuolization +	N	N

Degree of change
N = normal, no changes.
+ = slight changes.
++ = moderate changes.
+++ = extensive changes.

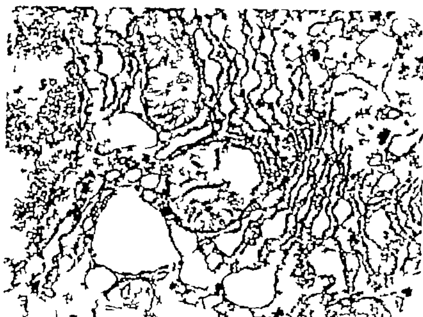


Fig. 2. Rat liver parenchymal cell two ho rs after administration of 500 mg/kg of Extractum filicis. Cisternae f the endoplasmic reticulum have become distended and multiple vacuoles appear in the cyt plasm. × 37 000

lar reticulum Glycogen disappeared in connection with the hypertrophy of the smooth membranes of the endoplasmic reticulum. Mitochondrial changes were rather small the mitochondria were not swollen but the internal cristae were sometimes distended (fig. 2) The Golgi area microbodies, lysosomes, nucleus, nucleolus and cell membrane exhibited no significant changes. The bile canaliculi and the space of Disse (peri sinusoidal space) also seemed to be unchanged The number of lipid inclusions was somewhat increased showing fatty infiltration of the parenchymal cells of the liver

Changes in the livers of the animals given 200 mg/kg and 50 mg/kg of the extract were essentially similar to those described above. The granular endoplasmic reticulum was decreased, the agranular endoplasmic reticulum was increased and there was no glycogen in the specimens of the livers of animals given 200 mg/kg. However the internal cristae of the mitochondria were not distended and the vacuolization of the cytoplasm was not so intensive as in the group receiving 500 mg/kg (fig. 3) In the group of animals given 50 mg/kg of filix extract the changes were even smaller The granular endoplasmic reticulum was only partly distended, the vacuolization of the cytoplasm was minute and the amount of glycogen had hardly decreased (fig. 4)

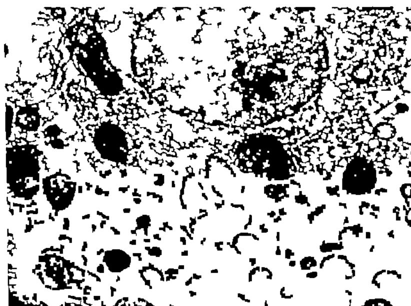


Fig. 3 Rat liver parenchymal cell two hours after administration of 200 mg/kg f Ex tractum filicis. Extensive disappearance of the granular endoplasmic reticulum. × 13 000.



Fig. 4. Rat liver parenchymal cell tw hours after administration of 90 mg/kg of Ex tractum filicis. There is no disappearance of glycogen and the endoplasmic reticul m bow only slight swelling. The bile canaliculi are normal. × 13,000



Fig. 5. Rat liver parenchymal cell seven days after administration of 200 mg/kg of Extractum filicis. The appearance of the cell is normal: there is abundant glycogen and the rough endoplasmic reticulum forms a network of narrow tubules. × 13,000.

In the specimens of the livers taken one day after a dose of 200 mg/kg there were changes of about the same magnitude as in the specimens taken two hours after the administration of filix extract. The nature of the changes was also the same. The specimens taken after three days showed only little change and after seven days they presented the picture of a normal rat liver cell with a network of narrow tubules of the endoplasmic reticulum and with large quantities of glycogen (fig. 5).

Discussion

In hepatic poisoning detectable morphological lesions of the hepatic cell are seen at an early stage in the endoplasmic reticulum and the ribosomes (REMMER & MERKER 1963 & 1965). This is probably due to the fact that in the liver the endoplasmic reticulum seems to be concerned with the detoxication mechanisms and with the lipid and the cholesterol metabolism (ROUILLER 1964). Depending on whether or not the ribosomes of the endoplasmic reticulum are attached to their external surface, there is a distinction between rough and smooth membranes (PALADE 1958 a & b). On the basis of functional differences these two categories of endoplasmic reticulum vary in their reciprocal proportions. The acute changes ob-

served in this study in the endoplasmic reticulum are similar to those observed after the administration of several toxic agents (for references see ROUILLER 1964) and must therefore be considered as a clear sign of the acute hepatocellular injury caused by the filix extract.

Mitochondrial lesions, enlargement of the nucleus, hypertrophy of the nucleolus, and breakdown of the cell membrane were not observed in this study except for rather small swellings of the internal cristae of the mitochondria after administration of 500 mg/kg of Extractum filicis. These changes presuppose other kinds of toxic mechanisms than that caused by Extractum filicis (for references see ROUILLER 1964).

Numerous hepatotoxic drugs induce fatty infiltration of the parenchymal cells i.e. fat droplets accumulate in the cytoplasm (ROUILLER 1964). This change was also seen to a minor extent in this study. Glycogen too responds very rapidly to acute liver injury and its concentration may already diminish 15–30 min. after the administration of a toxic agent (KORPASSY 1961). Glycogen depletion associated with the hypertrophy of the smooth membranes was distinctly seen in this study. This phenomen supports the view that hepatic cells have lost the capacity to metabolize glycogen in a normal manner (ORR *et al.* 1948).

The changes observed in the fine structure of the liver cell after administration of the filix extract show that this substance is a hepatotoxic substance (POPPER & SCHAFFNER 1959, KLATSKIN 1960). The pathological changes seem to be in proportion to the dose of the extract. These changes, however, are reversible and disappear completely in a few days. The observations made in the rats correlate well with the clinical observations which showed that the acute pathological liver function tests return to normal within some days (TAKKI 1967, TAKKI unpublished 1967).

Summary

The effect of different doses of Extractum filicis (Pharmacopoeia Fennica VII, 1956) on the fine structure of the rat liver was studied by means of the electron microscope.

The liver specimens revealed a relative increase of the agranular and a decrease of the granular endoplasmic reticulum. Cisternae of the granular endoplasmic reticulum were greatly distended, there were few ribosomes and multiple vacuoles appeared in the cytoplasm. Disappearance of glycogen was associated with the hypertrophy of the smooth membranes of the endoplasmic reticulum. The number of lipid inclusions also increased showing a fatty infiltration of the parenchymal cells.

The changes observed indicate hepatocellular damage and fulfil the criteria of a hepatotoxic agent.

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Further Studies on Chemically Induced Seizures and their Antagonism by Anticonvulsants During Postnatal Development in the Mouse

By

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(Received July 5 1967)

Studies on the effect of convulsant drugs during postnatal development are of interest since they can contribute to the understanding of the process of maturation in the brain. From the pharmacotherapeutic point of view such studies can also be regarded as a prerequisite for studies of the action of anticonvulsant drugs during development. Among the convulsants studied more thoroughly during development in animals are pentetrazol in cats (Cadilhac, Passouant Fontaine, Milhailoric & Poussant 1960), strychnine and brucine in rats (Pylkkö & Woodbury 1961), begremide in rats (Vulpe, Caldwell & Rodin 1963), pentetrazol in mice (Kobayashi, Inman Buno & Himwich 1963 Ferngren 1965), dimefline in rats (Setnikar & Magistretti 1964) and hexadifluorethyl-ether in rats (Webb & Davis 1964). Examples of greater sensitivity (strychnine) as well as of greater resistance (pentetrazol) to the convulsant action of the above-mentioned drugs have been found in newborn and very young animals as compared with that in older animals.

The action of anticonvulsant drugs during postnatal development has been the subject of rather few studies. The antagonizing effect against electrically induced seizures at selected ages in rats apart from the neonatal period, has been studied by Vernadakis & Woodbury (1965) for diphenylhydantoin and by Petty & Karler (1965) for acetazolamide, sulphanilamide, phenobarbital, diphenylhydantoin and carbon dioxide. The effects of phenobarbital and mephobarbital against pentetrazol induced seizures in developing mice from birth, have been studied previously by the present investigator (Ferngren 1965).

The convulsant drug picrotoxin which acts mainly by blocking presynaptic inhibition in the spinal cord (ECCLES 1965) does not seem to have been studied during development and was therefore included in the present study both for its own effect and for its antagonism by phenobarbital. The effect of trimethadione and its main metabolite dimethadione was studied against pentetrazol induced seizures, as these drugs have been widely used clinically against petit mal and are technically suitable since they are readily soluble in water and in addition are believed to have a specific action against pentetrazol (MILLICHAP 1965).

Materials and Methods

The experiments were performed on mice of the NMRI strain. About 75 young animals were used (only once) for each age group and drug. The animals were divided into 3–5 dose groups and given doses of the convulsant or anticonvulsant in geometrical progression.

The following age groups were studied 1 3 5 9 21 days old (with no separation of sexes) and adult male mice. In some additional experiments 12 and 30 days old mice of both sexes were also studied. The reason for this was that ontogenic analysis of reflexes and behaviour in the mouse has suggested five periods of neurological development birth-3 days, 3–9 days, 9–15 days, 15–26 days, 26 days to maturity (FOX 1965)

Seizures were defined as tonic or clonic-tonic convulsions lasting for at least 5 seconds. The median convulsant and anticonvulsant doses (for definitions see FERNGREN 1965) were calculated with 95% fiducial limits by probit analysis (Ph. Nord Vol. IV) The anticonvulsant drug was injected subcutaneously and dorsally 30, 50 or 90 minutes after 1.5 times the CD50 for the convulsant and the age group. The handling of the animals and the method of injection was the same as that described previously (FERNGREN 1965). In the present study a total number of more than 7 800 mice was used.

Test solutions

1 *Pentetrazol.* A 10% (w/v) solution in 0.9% (w/v) NaCl.
2. *Picrotoxin.* A 0.1% (w/v) solution in distilled water or 0.9% NaCl.
3. *Phenobarbital* – phenemalum NFN – (5-ethyl 5-phenylbarbituric acid) A 1% (w/v) solution of the sodium salt in 0.9% NaCl was used for all age groups except the 1-day-old animals which received a 0.2% solution in order to get more suitable volumes for injection.
4 *Trimethadione* – trimethadionum NFN – (3,5,5-trimethyl-2,4-oxazolidinedione)[1]. A 5% (w/v) solution in 0.9% NaCl.
5 *Dimethadione* – dimethadionum NFN – (5,5-dimethyl-2,4-oxazolidinedione)[2]. A 5% (w/v) solution in 0.9% NaCl.

1) Supplied through the courtesy of the representative of Abbott Laboratories in Scandinavia.
2) Supplied through the courtesy of Dr Robert L. Alberti, Abbott Laboratories, North Chicago



Fig. 1 Median convulsant doses (mg/kg) for picrotoxin (●) jected subcutaneously int developing mice. Theoretical CD50:s (⊙) calculated from the mean body area for each age group in relation to the median convulsant dose and mean body area for adult males are included for comparison. Vertical bars indicate 95% fiducial limits.

Results

Picrotoxin

Figure 1 shows the median convulsant doses of picrotoxin in mice during postnatal development. The figure also includes the theoretically expected values according to the surface area principle for dose reduction from adult to younger animals. (Calculations based on Dawsons formula, see FERNGREN 1965). The median convulsant dose increases from birth to 3 days and then decreases to the age of 12 days, from which age it

Table 1

Statistical comparison of CD50 for picrotoxin in developing mice (Student t-test).

Compared age groups	P
1 3	<0.001
3–5	<0.05
5–9	<0.001
9–12	>0.05
12 21	<0.001
21 30	<0.001
30–adult.	>0.05

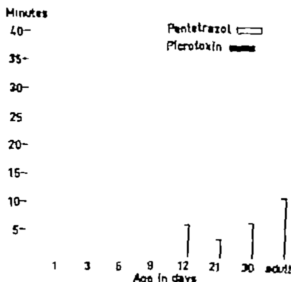


Fig. 2. Mean induction time from subcutaneous injection to the appearance of seizures in minutes for pentetrazol- and picrotoxin-induced seizures in developing mice. Each bar represents at least 20 animals.

starts to increase again Table I shows a comparison of the CD50 s at various ages. It can be seen from the table that the difference between 3- and 5-day-old mice is probably significant while that between 9- and 12-day-old is not significant.

The mean induction time for defined seizures (the whole convulsive episode lasting more than 5 seconds) varies with age as seen in fig. 2. The seizures come on most quickly in 21 and 30-day-old mice and the longest latency is seen in newborns. In 3-day-old mice the induction time is significantly shorter than in newborns and 5-day-old mice. The pattern of seizures is similar to that seen with pentetrazol-induced seizures, but the different phases of the seizures are of longer duration. In newborn and very young mice the convulsions consisted of tonic extension backwards of the forelimbs and urination. Clonic episodes are seen from the age of 12 days in some animals, and consistently in 21-day-old animals.

Phenobarbital-picrotoxin

The median anticonvulsant doses during postnatal development of phenobarbital against picrotoxin induced seizures for three intervals between the injections (30 50 and 90 minutes) are presented in fig. 3 For each interval and age theoretical ED50 s calculated from dose regression

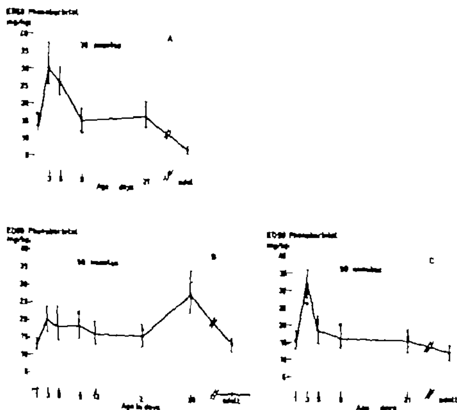


Fig. 3 Median anticonvulsant doses (mg/kg) for phenobarbital (●) injected subcutaneously against picrotoxin-induced seizures in develop g mice. The interval between the injections of the two drugs is 30 minutes (A), 50 minutes (B) and 90 minutes (C). Theoretical ED50 values (○) calculated from the mean body area for each age group and interval in relation to the median anticonvulsant dose and mean body area for adult males are included for comparison. Vertical bars indicate 95% fiducial limits

after body area in comparison with the adult male ED50 are included. Regardless of interval and age the ED50 is mainly of the order of 10–20 mg/kg. Table 2 shows a comparison between the ED50 s of various ages for each injection interval. In adult males the lowest ED50 is found 30 minutes after the injection and at this interval, the difference in ED50 between 1-day-old and 3-day-old, as well as that between 5-day-old and 9-day-old mice is highly significant.

At the middle injection interval, 12-day-old and 30-day-old mice were also studied. The difference in median anticonvulsant dose between 30-day-old and 21-day-old and between 30-day-old and adult male mice is highly significant. The ED50 in 12-day-old mice does not differ signif

Table 2

Statistical comparison of ED50 s for phenobarbital against picrotoxin-induced seizures in developing mice (Student's t-test).

Compared age groups	Injection interval		
	30 min.	50 min.	90 min.
1–3	$P < 0.001$	$P < 0.001$	$P < 0.001$
3–5	$P > 0.05$	$P > 0.05$	$P < 0.001$
5–9	$P < 0.001$	$P > 0.05$	$P > 0.05$
9–12	–	$P > 0.05$	–
9–21	$P > 0.05$	$P > 0.05$	$P > 0.05$
12–21	–	$P > 0.05$	
21–30	–	$P < 0.001$	–
21–adult	$P < 0.001$	$P > 0.05$	$P > 0.05$
30–adult	–	$P < 0.001$	–

icantly from that of 9-day-old or 21-day-old animals. At the 90 minutes injection interval only the ED50 in 3-day-old mice differs significantly from the others, and is even higher than what would be expected from the body area.

Trimethadione-pentetrazol and dimethadione-pentetrazol

The anticonvulsant activity of trimethadione and its main metabolite dimethadione during postnatal development in the mouse against pentetrazol-induced seizures is presented in fig. 4 Table 3 shows a comparison between the ED50 s of trimethadione and table 4 gives a comparison between the ED50 s of dimethadione Usually the median anticonvulsant doses of trimethadione are lower than those of dimethadione In 9-day-old animals, however the ED50 s for the two drugs are about the same at all intervals studied This was also found in adult males at the shortest interval.

The effect of trimethadione has different developmental patterns at all intervals studied The high ED50 in 3-day-old animals at the longest interval is striking and its difference from 1-day-old and 5-day-old mice is highly significant. It should be noted that the corresponding ED50 for dimethadione has the same order of magnitude at this interval. The trimethadione ED50 s for newborns are also rather low at the two longer intervals.

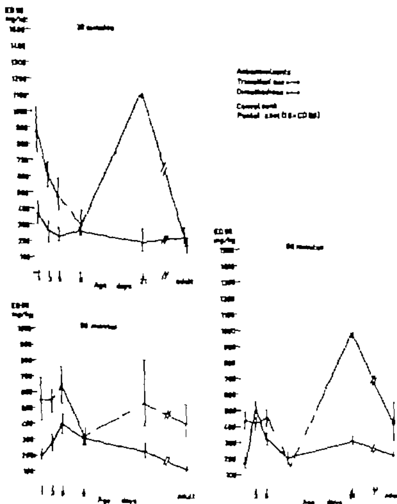


Fig. 4 Median anticonvulsant doses (mg/kg) for trimethadione (●) and dimethadione (○) injected subcutaneously against pentetrazol-induced seizures in developing mice. The interval between the injection of the anticonvulsant and the convulsant drug is 30 minutes (A), 50 minutes (B) and 90 minutes (C). Vertical bars indicate 95 % fiducial limits.

On the other hand, the effect of *dimethadione* has in its developmental pattern some similarities at all intervals studied particularly from the age of 5 days. A low ED50 is thus found in 9-day-old and in adult male mice, while a very high ED50 is found in 21-day-old animals. The difference between the ED50 s of 9-day-old and 21-day-old animals at the middle interval is probably significant, while the difference between 21-day-old and adult males is not significant.

Table 3

Statistical comparison of ED50 s for trimethadion against pentetrazol-induced seizures in developing mice (Student s t-test).

Compared age groups	Injection interval		
	30 min.	50 min.	90 min.
1–3	$P < 0.05$	$P \sim 0.01$	$P < 0.001$
3–5	$P > 0.05$	$P < 0.01$	$P < 0.001$
5–9	$P > 0.05$	$P < 0.05$	$P < 0.001$
9–21	$P > 0.05$	$P < 0.05$	$P < 0.001$
21–adult	$P > 0.05$	$P < 0.001$	$P < 0.001$

Table 4

Statistical comparison of ED50 s for dimethadione against pentetrazol induced seizures in developing mice (Student's t-test).

Compared age groups	Injection interval		
	30 min.	50 min.	90 min
1–3	$P \sim 0.001$	$P > 0.05$	$P > 0.05$
3–5	$P < 0.05$	$P > 0.05$	$P > 0.05$
5–9	$P < 0.01$	$P < 0.001$	$P < 0.001$
9–21	$P < 0.001$	P 0.05	$P < 0.001$
21–adult	$P < 0.001$	$P > 0.05$	$P < 0.01$

Discussion

Picrotoxin

The postnatal developmental pattern in the mouse for the convulsant effect of picrotoxin is different from the developmental pattern of the effect of pentetrazol, a drug studied previously at this laboratory The highest CD50 for pentetrazol was found in newborns but the highest CD50 for picrotoxin was found in 30-day-old animals. In mice younger *than 30 days* the *CD50 s for picrotoxin* were much *lower* than would be expected from their body area. The CD50 for newborns is about *half* the adult male value in mg/kg. This has also been found in rats for another convulsant drug, strychnine, where the CD50 in newborn animals was about eight times less than the adult value (PYLKKÖ & WOODBURY 1961).

However the seizure pattern showed the same main characteristics for picrotoxin as for pentetrazol.

One reason for the differences found in the convulsant effect during postnatal development for pentetrazol and picrotoxin could be differences in absorption, distribution and penetration of the blood brain-barrier. The induction time for seizure, however shows the same variation as maturation develops both for picrotoxin and for pentetrazol, the only difference being that at all ages picrotoxin induced seizures start about 10 minutes later than pentetrazol-induced seizures. This fact indicates that there is a difference between the drugs studied as regards their penetration into the brain, but this difference seems to be independent of maturation. Because methods of evaluating these drugs in biological material are not available further analysis of their respective fates in the body has not been possible.

The different patterns in convulsant effect of pentetrazol and picrotoxin during development may also be explained on the basis that the convulsants have different mechanisms of action and that the structures on which the drugs act have different postnatal development. There is some experimental evidence for this possibility. ECCLES, SCHMIDT & WILLIS (1963) reported that picrotoxin depresses presynaptic inhibition in the spinal cord in subconvulsive doses while pentetrazol has no such effect. Further KURIYAMA, ROBERTS & RUBINSTEIN (1966) have shown in adult mice that an increase in brain levels of gamma-aminobutyric acid antagonizes pentetrazol-induced seizures but not picrotoxin- or strychnine induced seizures.

Phenobarbital-picrotoxin

The anticonvulsant effect of phenobarbital against picrotoxin-induced seizures during postnatal development is different in the neonatal period for each injection interval, and similar from the age of 9 days independent of the interval. It is evident from fig. 3 that at none of the intervals studied did the developmental pattern of the anticonvulsant effect follow the body area principle for dose reduction from adult to younger animals. The patterns at these two periods before and after 9 days can be compared with the results for the action of phenobarbital against pentetrazol-induced seizures which have been reported previously (FERNGREN 1965).

Such a comparison shows the following. At the shortest interval (fig. 3A) in the period less than 9 days, the effect of phenobarbital against picrotoxin shows a similar pattern as that against pentetrazol with higher ED50 s in 3- and 5-day-old mice. But at the other intervals no such similarities appear. The results in 3-day-old animals at the 90 minutes

injection interval where a high ED50 was found for phenobarbital-picrotoxin but not for phenobarbital-pentetrazol (FERNGREN 1965), may be due to the fact that picrotoxin-induced seizures at this age come on about 15 minutes later than pentetrazol seizures. This would leave more time for the anticonvulsant to disappear from the brain. As the more detailed mechanism of action for the anticonvulsant effect of phenobarbital is unknown, no explanation can be offered for the difference found at the period from birth to 9 days of age.

For the period after the age of 9 days the high ED50 in 30-day-old mice at the middle interval, may be related to the high CD50 for picrotoxin at this age The peak effect of phenobarbital against picrotoxin-induced seizures is almost 30 minutes for adult male mice. At all the other age groups and intervals, higher doses of phenobarbital are needed to antagonize picrotoxin-induced than pentetrazol induced seizures. This finding also supports the view that there is a difference in the mechanisms of action of these two convulsant drugs.

Trimethadione-pentetrazol and dimethadione-pentetrazol

The anticonvulsant effect of trimethadione on chronic administration has been attributed to its conversion to the N-demethylated derivative dimethadione (BUTLER & WADELL 1958 CHAMBERLAIN, WADELL & BUTLER 1965) On the other hand the results of the present study show that on acute administration to mice during development, the anticonvulsant effect of dimethadione is generally less than that of trimethadione On the basis of these experiments the theory that trimethadione acts only after demethylation to dimethadione can be questioned. Only in 9-day-old mice is the effect of dimethadione at all intervals as good as that of trimethadione. Before this age, it is only in 3-day-old animals (90 minutes interval) that the ED50 of dimethadione is similar to that of trimethadione. It seems possible that the high ED50 of trimethadione at this age group depends on a more rapid conversion to dimethadione.

It can be postulated from the results obtained after the age of 9 days, that the different physico-chemical properties of the drugs become more decisive as maturation proceeds. According to BUTLER, WADELL & POOLE (1965) trimethadione is a molecule that cannot be dissociated while dimethadione is 95 / ionized at pH 7 4 This difference has consequences both with regard to the penetration of the blood-brain-barrier and the excretion through the kidneys. Dimethadione is said to be eliminated as such and has a pH-dependent renal excretion. Physiological conditions such as a higher intracellular pH might make the penetration into the brain easier in 9-day-old mice. The high ED50 of dimethadione

in 21-day-old animals, at the longest interval, may be due to a rapid excretion possibly related to the urinary pH

The findings in 3-day-old animals with a rapidly vanishing effect of phenobarbital against picrotoxin and of trimethadione against pentetrazol, seem to indicate a common factor which could be related to some aspect of the bio-transformation of the drug at this age. The peak plasma levels of corticoids as reported in previously handled 3-day-old rats after stress (Levine & Mullins 1966) may have a counterpart in mice, and also be of importance in this connection.

Summary

The convulsant effect of picrotoxin the anticonvulsant effect of phenobarbital against picrotoxin and the anticonvulsant effect of trimethadione and dimethadione against pentetrazol were studied in mice during post natal development, by dermination of median effective doses at the following ages 1 3 5 9 and 21 days old, adult males, and in some cases 12 and 30-day-old mice. Picrotoxin had a developmental pattern with low CD50 s from 1-day-old to 12-day-old and higher CD50 s during later development while the opposite maturational variation was found previously for pentetrazol. The seizure pattern was similar for both drugs. The developmental variation in the effects of the anticonvulsant drugs was found to be dependent on the interval between the injection of the anticonvulsant and the convulsant drug. Striking differences were found in the effects of the structurally related drugs dimethadione and trimethadione. Dimethadione was found to be less active against pentetrazol-induced seizures at all ages except in 9-day-old animals, where the activity was the same as for trimethadione. For trimethadione and phenobarbital the effect disappeared more rapidly in 3-day-old than in 1-day-old or 5-day-old mice.

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Metabolism of Phenolic Acids by the Rat Intestinal Microflora

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(Received September 2, 1967)

The metabolism of several phenolic acids by intestinal contents has been reported by BOOTH & WILLIAMS (1963a & b). These workers found that caffeic acid (3 4-dihydroxycinnamic acid) was reduced and dehydroxylated when incubated with the intestinal microorganisms. It was also shown that protocatechuic acid (3 4-dihydroxybenzoic acid) was decarboxylated to catechol and homoprotocatechuic acid (3 4-dihydroxyphenylacetic acid) dehydroxylated to *m*-hydroxyphenylacetic acid

In a recent report from this laboratory (SCHELINE 1966a) it was shown that protocatechuic acid and gallic acid (3 4,5-trihydroxybenzoic acid) were decarboxylated when administered orally to rats and that this reaction was carried out by the intestinal microflora. Furthermore, it was found that several phenolic benzoic acid derivatives containing a free *p*-hydroxyl group were decarboxylated by rat caecal microflora (SCHELINE 1966b). It was also found that some compounds containing a methoxyl group underwent demethylation We have recently reported (SCHELINE 1967) that homoprotocatechuic acid is decarboxylated to 4-methylcatechol by rat caecal microflora and that this metabolite is excreted in the urine of rats following oral administration of homoprotocatechuic acid

These results clearly indicate that the intestinal microflora may be of considerable importance in determining the metabolite fate of phenolic acids. It was therefore deemed worthwhile to extend this study to include other phenolic phenylacetic acids as well as phenolic phenylpropionic and cinnamic acids. The present report describes the metabolism of a number of these compounds by rat caecal microflora and also discusses the role of the intestinal flora in the metabolism of caffeic acid when administered to rats.

Methods

Animals

Male albino rats, weighing from 300–350 g, were used. They were maintained on a commercial pellet diet (Felleskjøpet, Oslo).

Diet

The purified diet contained sucrose, 680 g, casein (British Drug Houses, Light White Soluble) 200 g, soya oil, 60 g, U.S.P. XIV salt mixture, 40 g, and a vitamin mixture 20 g. The vitamin mixture contained thiamine hydrochloride, 25 mg, riboflavine 22 mg, pyridoxine hydrochloride, 22 mg, calcium pantothenate, 33 mg, nicotinamide, 22 mg, ascorbic acid, 330 mg, vitamin A 5000 I.U., vitamin D_2, 600 I.U., tocopherol acetate, 51 mg in sufficient glucose to make 20 g.

Compounds

3-(3-Hydroxy-4-methoxyphenyl) propionic acid (m.p. 144°) and 3-(4-hydroxy-3-methoxyphenyl)propionic acid (m.p. 88°) were prepared from isoferulic and ferulic acids, respectively by hydrogenation with platinum oxide catalyst. 3-Hydroxyphenylacetic acid (m.p. 127–128°), 3-(3-hydroxyphenyl)propionic acid (m.p. 110–110°) and 4-ethylcatechol (m.p. 37–38°) were obtained from their respective methyl ethers, by treatment with hydrobromic or hydriotic acids. 3-Hydroxy-4-methoxyphenylacetic acid (m.p. 127–128°) and 4-hydroxy-3-methoxyphenylacetic acid (m.p. 141–142.5°) were prepared by the methods of GRUNDON & PERRY (1954) and FISHER & HIBBERT (1947), respectively. 4-Vinylguaiacol was prepared in poor yield from ferulic acid by decarboxylation under heating at reduced pressure. It was reduced with platinum oxide catalyst to give a small amount of 4-ethylguaiacol. Other compounds were obtained commercially. They were checked for purity chromatographically and recrystallized if required.

Incubation with caecal extracts

The incubation medium consisted of 0.5% glucose, 0.5% yeast extract (Difco) and 0.5% peptone (Difco) in 0.1 M phosphate buffer (pH 7.4). Unless otherwise stated the test substance (5–10 mg) in medium (10 ml) was incubated for 22 hours at 37° with rat caecal extracts as described previously (SCHELINE 1966b). The samples were then acidified with concentrated hydrochloric acid (1 ml) and extracted with three 25 ml portions of ether. The ether extracts were dried over anhydrous sodium sulphate, evaporated and the residues dissolved in 1 ml acetone. Controls were prepared in the same way except that the caecal extract was omitted.

Animal experiments

A few preliminary experiments were carried out on rats fed the commercial diet, but otherwise the animals were given the purified diet (20 g/day) beginning two days before dosing. The rats had free access to drinking water.

Test substances were administered in doses of 100 or 500 mg/kg as an aqueous suspension (1–2 ml) by stomach tube or given in a dose of 100 mg/kg as a solution of their sodium salts (1–2 ml) by intraperitoneal injection to rats under light ether anaesthesia. The urine and faeces were collected separately in containers placed in solid carbon dioxide (SCHELINE & LONGBERG 1965).

After thawing and filtering the 24 and 48 hour urine samples were diluted to 20–25 ml

and extracted with three 25 ml portions of ether after adjusting to pH ~3 with hydrochloric acid. The ether was removed from the aqueous phase by slight warming under reduced pressure and the pH was adjusted to 7.0 by the addition of Na_2HPO_4 solution. β-Glucuronidase (approx. 3,000 units, Sigma Chemical Co., Type I) and a drop of chloroform were added and the solution incubated at 37° for 2 hours. After acidificatio and ether extraction as described above, the ether solutions were dried over anhydrous sodium sulphate, evaporated and the residues dissolved in 1 ml acetone. The two ether extracts are respectively called "free" and "bound".

Biliary studies were carried out on rats anesthetised with nembutal sodium (pentobarbitone) by placing a thin plastic tube in the common bile duct. The animals were placed in a restraining cage (SCHILLER 1965) where they had access to food and water and the bile was collected at 0°. Ether extraction of these samples was carried out as described above for the urine samples except that 1500 units of β-glucuronidase were used.

Biliary excretion was prevented by tying off the common bile duct at two points and severing the duct between these points.

Chromatography

The above acetone extracts, together with appropriate standards, were examined by thin-layer chromatography on 0.5 mm thick layers of cellulose. Sigmacell Type 19 (Sigma Chemical Co.) was used with solvent 1 (benzene-glacial acetic acid-H_2O (6 : 7 : 3) upper layer)) and MN 300 (Macherey, Nagel and Co.) was used with solvent 2 (20% aqueous potassium chloride-glacial acetic acid (100 : 1)). Rf values, colour reactions after spraying with fast blue B salt followed by saturated $NaHCO_3$ solution and fluorescence under ultraviolet light are shown in table 1.

Characterization of decarboxylated metabolites

The acetone solutions from the *in vitro* samples were dissolved in ether (25 ml) and extracted twice with equal volumes of 5% N HCO_3. After washing with 0.1 N-HCl (approx. 10 ml), the ether was dried over anhydrous sodium sulphate. The ether was then evaporated and the residue taken up in water. The ultraviolet absorption and fluorescence measurements of these solutions were determined by means of a Beckman DB spectrophotometer and an Aminco-Bowman Spectrophotofluorometer respectively. Infrared spectra (4000–650 cm⁻¹) of the substances in KBr pellets were determined with a Hilger H-900 spectrophotometer.

Results

Metabolism by intestinal microflora

The results of the experiments in which the phenolic acids were incubated with rat caecal extracts are shown in table 2. Under these conditions, four reactions were found: reduction of a double bond, dehydroxylation, decarboxylation and demethylation.

Reduction of a double bond was observed in all the cinnamic acid derivatives tested. The reduced metabolites were major metabolites of these compounds and were found in all the samples except for those of caffeic acid where hydrocaffeic acid was detected in six out of 14 experiments.

Table 1

Thin-layer chromatography and colour reactions of some phenolic compounds.

Compound		Rf		Colour with fast blue B salt	Fluorescence 254 mμ
Chemical name	Trivial name	Solvent 1	Solvent 2		
2-Hydroxyphenylacetic acid	–	0.37	0.77	red	–
3-Hydroxyphenylacetic acid		0.24	0.74	red-orange	–
4-Hydro yphenylacetic acid	–	0.2.	0.72	brown	–
3-(2-Hydroxyphenyl)-propionic acid	Melilotic acid	0.66	0.63	red	–
3-(3-Hydroxyphenyl)-propionic acid	m-Hydroxyphenyl propionic acid	0.39	0.61	red-orange	–
3-(4-Hydroxyphenyl)-propionic acid	Phloretic acid	0.41	0.61	brown	–
2-Hydroxycinnamic acid	o-Coumaric cid.	0.39	0.23	purple-red	blue whit
3-Hydroxycinnamic acid	m-Coumaric acid	0.32	0.23	red	blue
4-Hydroxycinnamic acid	p-Coumaric acid	0.29	0.19	purple	blue-purple?)
2,5-Dihydroxyphenylacetic acid	H mogentisic acid	0.03	0.69	dark brown	–
3,4-Dihydroxyphenylacetic acid	H moprotocatechuic acid	0.03	0.68	dark brown	–
3-(3,4-Dihydroxyphenyl)-propionic acid	Hydrocaffeic acid	0.05	0.56	pi k brown	–
3,4-Dihydroxycinnamic acid	Caffeic acid,	0.04	0.15	green-grey	–
3-Hydroxy-4-methoxyphenylacetic acid	Homoisovanillic acid	0.55	0.68	purple-red	–

4-Hydroxy-3-methoxyphenylacetic acid	Homovanillic acid	0.61	0.68	brown	
3-(3-Hydroxy-4-methoxyphenyl)-propionic acid	Hydroisoferulic acid	0.78	0.55	purple-red	–
3-(4-Hydroxy-3-methoxyphenyl)-propionic acid	Hydroferulic acid	0.77	0.38	brown	–
3-Hydroxy-4-methoxycinnamic acid	Isoferulic acid.	0.61	0.11	purple	blue-p rple
4-Hydroxy-3-methoxycinnamic acid	Ferulic acid	0.75	0.16	purple	blue
2-Hydroxytoluene	o-Cresol	0.98	–	orange	
3-Hydroxytoluene	m-Cresol	0.95		red-orange	–
4-Hydroxytoluene	p-Cresol	0.95	–	yellow-brown	–
1-Ethyl-4-hydroxybenzene.	p-Ethylphenol	0.97		yellow-brown	–
4-Hydroxystyrene	p-Vinylphenol	0.92[1])	0.32[1])	purple-brow	purple
1,2-Dihydroxy-4-methylbenzene.	4-Methylcatechol	0.43	0.52	grey-violet	–
1,2-Dihydroxy-4-ethylbenzene	4-Ethylcatechol	0.61	0.48	grey-violet	–
3,4-Dihydroxystyrene	4-Vinylcatechol	0.42[1])	0.39[1])	grey-purple	purple
4-Ethyl-2-methoxyphenol	4-Ethylguaiacol	0.98		brown	
4-Hydroxy-3-methoxystyrene	4-Vinylguaiacol.	0.98		purple-brown	purple

1) Values of metabolites. 2) After exposure to NH_3 vapour

Table 2

Metabolism of phenolic compounds by the rat intestinal microflora.

Test substance (5–10 mg) incubated 22 h. with rat caecum extract (1 ml) in 0.1 M pH 7.4 phosphate-glucose-peptone-yeast extract medium (10 ml).

Compound	No of experiments	Observations
2-Hydroxyphenylacetic acid	3[1]	Only unchanged compound
3-Hydroxyphenylacetic acid	6	Only unchanged compound
4-Hydroxyphenylacetic acid	8	Unchanged compound in 8/8, *p*-cresol in 4/8
Melilotic acid	3	Only unchanged compound
m-Hydroxyphenylpropionic acid	3	Only unchanged compound
Phloretic acid	5	Only unchanged compound
o-Coumaric acid	3	Unchanged compound in 3/3, melilotic acid in 3/3
m-Coumaric acid	3	Unchanged compound in 3/3, *m*-hydroxyphenylpropionic acid in 3/3
p-Coumaric acid	5	No unchanged compound, phloretic acid in 5/5, 4-vinylphenol in 4/5, 4-ethylphenol in 1/5
Homogentisic acid	3	Only unchanged compound
Homoprotocatechuic acid	6	Unchanged compound in 6/6, *m*-hydroxyphenylacetic acid in 6/6, 4-methylcatechol in 3/6
Hydrocaffeic acid	6	Small amount of unchanged compound in 2/6, *m*-hydroxyphenylpropionic acid 6/6
Caffeic acid	15	Small amount of unchanged compound in 9/15, hydrocaffeic acid in 7/15, *m*-hydroxyphenylpropionic acid in 15/15, 4-vinylcatechol in 5/15, 4-ethylcatechol in 13/15
Homoisovanillic acid	3	Unchanged compound in 3/3, small amount of homoprotocatechuic acid in 1/3
Homovanillic acid	5	Unchanged compound in 5/5, homoprotocatechuic acid in 2/5, *m*-hydroxyphenylacetic acid in 2/5, 4-methylcatechol in 2/5
Hydroisoferulic acid	2	Unchanged compound in 2/2, *m*-hydroxyphenylpropionic acid in 2/2
Hydroferulic acid.	3	Unchanged compound in 3/3, *m*-hydroxyphenylpropionic acid in 3/3
Isoferulic acid	4	Unchanged compound in 4/4, hydroisoferulic acid in 4/4, small amount of *m*-hydroxyphenylpropionic acid in 4/4
Ferulic acid	6	Small amount of unchanged compound in 3/6, hydroferulic acid in 6/6, *m*-hydroxyphenylpropionic acid in 6/6, hydrocaffeic acid in 2/6, 4-ethylcatechol in 3/6, 4-vinylguaiacol in 3/6

[1]) Equal to number of caeca used.

All the compounds containing a 3 4-dihydroxyphenyl group underwent dehydroxylation. Dehydroxylation occurred in the *p*-position resulting in *m*-hydroxy derivatives and no *p*-hydroxy compounds were detected under the present experimental conditions. As 4-hydroxyphenylacetic acid was often detected in varying amounts in the caecal incubates with or without added test substance no assessment of its formation from homoprotocatechuic acid in the present experiments can be made At most, it could only have been formed in small amounts. In the case of caffeic acid, dehydroxylation could lead to *m*-coumaric acid or following reduction of the double bond, to *m*-hydroxyphenylpropionic acid. The latter metabolite was detected in all the caffeic acid experiments but *m*-coumaric acid was never detected A further experiment was carried out in which caffeic acid was incubated with caecum extract for 2, 7 10 22 and 46 hours. *m*-Coumaric acid was not found in any of these samples although *m*-hydroxyphenylpropionic acid was found in the 10-hour sample and subsequently No attempt was made to investigate the possibility of dehydroxylation of the monohydroxy or hydroxy-methoxy compounds by caecal extracts. Such metabolites would not be detected with the fast blue B salt reagent. 2,5-Dihydroxyphenylacetic acid (homogentisic acid) was not dehydroxylated in these experiments.

Decarboxylation of the compounds studied was found to occur only when a free *p*-hydroxyl group was present. Except for homovanillic acid, this reaction was seen with both the phenylacetic and cinnamic acid derivatives. However the phenolic phenylpropionic acids containing a free *p*-hydroxy group (phloretic, hydrocaffeic and hydroferulic acids) were found to be resistent to decarboxylation This indicates that decarboxylation of the cinnamic acid derivatives resulted in the formation of the vinyl metabolites which then underwent reduction to the corresponding ethyl compounds This sequence was confirmed in an experiment in which *p*-coumaric and caffeic acids were incubated with caecum extract for 2, 7 10 22 and 46 hours. The 2 hour samples showed only unchanged compounds while those taken after 7 hours showed 4-vinylphenol and 4-vinylcatechol, respectively as the only decarboxylated metabolites. After 10 hours the *p*-coumaric acid sample still showed only 4-vinylphenol but both 4-vinylcatechol and 4-ethylcatechol were detected in the caffeic acid sample Only the ethyl derivatives were found in the 22 hour and 46 hour samples.

The identities of the decarboxylated metabolites were confirmed by comparing their ultraviolet absorption and fluorescence spectra with authentic samples or with values reported in the literature. These data are shown in table 3 Two of the 4-hydroxyphenylacetic acid samples which showed the presence of a metabolite corresponding chromatographically

Table 3

Ultraviolet absorption and fluorescence maxima of some substituted phenols and decarboxylated metabolites of phenolic acids.

Compound	Ultraviolet absorption maxima in H_2O	Ultraviolet absorption maxima in 95% ethanol	Fluorescence maxima in H_2O
	mμ	mμ	mμ
p-Cresol	276	–	309
4-Hydroxyphenylacetic acid phenolic metabolite	276	–	309
4-Vinylphenol	–	259[1]	–
4-Ethylphenol	276	–	308
p-Coumaric acid phenolic metabolite A	257	259	330
p-Coumaric acid phenolic metabolite B	276	–	308
4-Vinylcatechol	258[1]	–	–
4-Ethylcatechol	279	–	321
Caffeic acid phenolic metabolite A	258	–	343
Caffeic acid phenolic metabolite B	279	–	321
4-Vinylguaiacol	258	–	339
4-Ethylguaiacol	279	–	319
Ferulic acid phenolic metabolite A	258	–	339

[1] Values reported by FINKLE *et al.* (1962).

to *p*-cresol were treated as described in the Methods section to remove acidic compounds. In both cases the spectral data of the metabolite were identical to those of *p*-cresol (table 3). When a sample obtained from incubating medium with caecum extract alone was similarly treated, no ultraviolet absorption maximum was found in the region of 220–320 mμ. On activation at 280 mμ, this sample showed a barely detectable fluorescence at approx. 350 mμ.

The vinyl metabolite (metabolite A) from the 22 hour incubates of *p*-coumaric acid was obtained similarly in two experiments. As an authentic sample of 4-vinylphenol was not available in the present study the metabolite was also dissolved in 95% ethanol so as to compare the absorption maximum with the value reported by FINKLE *et al.* (1962). They also found that this substance when chromatographed on Whatman No. 1 paper gave Rf values of 0.65 in 4% acetic acid and 0.95 in chloroform-acetic acid-water (2:1:1 lower phase). The values for the *p*-coumaric acid meta-

bolite A obtained in the present experiments when chromatographed in the same way were found to be 0 68 and 0.98 respectively

Only one of the *p*-coumaric acid samples (table 2) was found to contain 4-ethylphenol. A large-scale experiment was therefore carried out with *p*-coumaric acid (250 mg) in medium (500 ml) which was incubated with caecum extract (10 ml) for 46 hours. After extraction with ether and 5% $NaHCO_3$, the phenolic residue was twice sublimated under reduced pressure to give a white, solid product. This compound (*p*-coumaric acid metabolite B) gave the spectral data shown in table 3 The infrared spectrum of this material was identical with that of 4-ethylphenol.

The metabolism of caffeic acid by caecal extracts resulted in the formation of caffeic acid phenolic metabolites A and B which were identified as 4-vinylcatechol and 4-ethylcatechol, respectively The former compound was isolated from a 22 hour incubate and gave an absorption maximum in water identical to that reported by FINKLE *et al.* (1962) (table 3). This metabolite was also chromatographed on Whatman No 1 paper and showed on Rf value of 0 61 in 4% acetic acid as compared with the reported value of 0 6. 4-Ethylcatechol was isolated from a large-scale experiment identical to that described above for *p*-coumaric acid Sublimation of the phenolic residue resulted in an oil which was recrystallized from a mixture of benzene-petroleum ether (60–80°) This substance gave the spectral data shown in table 3 for caffeic acid phenolic metabolite B Its infrared spectrum was identical to that of 4-ethylcatechol

Identification of the decarboxylated metabolites of ferulic acid proved difficult for several reasons. One factor is the limited conversion to decarboxylated metabolites due to the multiplicity of reactions undergone by ferulic acid. It is also likely that both 4-vinylguaiacol and 4-ethylguaiacol are not end-products of metabolism as they can be metabolized further (e.g. by demethylation). Finally the chromatographic solvents used do not allow separation of the two guaiacol metabolites. Extraction of some of the ferulic acid incubates for phenols, however has given spectral data confirming the presence of 4-vinylguaiacol. A large-scale experiment similar to the ones mentioned above (46 hour incubation) was carried out and, after chromatography on cellulose layers in solvent 1 the band at the solvent front was eluted with water The ultraviolet absorption and fluorescence maxima coincided with those of 4-vinylguaiacol, although impurities were present. Further chromatography on silicagel G (Merck) in benzene gave a band at approx. Rf 0.3 which, based on spectral data, was found to be 4-vinylguaiacol. 4-Ethylguaiacol was not detected in the ferulic acid incubates.

Incubation of the test compounds in the absence of caecal extract resulted in the detection of only the unchanged compounds. This was

the case when caecal extract which had been autoclaved at 121 for 15 min. was added. However previous heating of caecal extract at 80 for 15 minutes abolished the decarboxylation of protocatechuic and homoprotocatechuic acids but not that of caffeic or ferulic acids. The incubates of these cinnamic acid derivatives contained both the unchanged compounds and their vinyl metabolites.

The present study included six methyl ethers and the results presented in table 2 show that they were all demethylated when incubated with rat caecal contents. It appears that the 3-methoxyl group undergoes cleavage somewhat more readily than the 4-methoxyl group as both homovanillic and ferulic acids were converted to demethylated metabolites to a greater extent than their isomers

Metabolism in rats

In order to investigate the role of the intestinal flora in the metabolism of the phenolic compounds following their administration to animals, caffeic acid was chosen as a model compound As the purpose of these experiments was to study the metabolites known to be formed by intestinal flora, no attempt was made to determine all the metabolites of caffeic acid.

Experiments on three rats fed the ordinary diet and two rats fed the purified diet showed that when caffeic acid (100 mg/kg) was administered orally *m*-hydroxyphenylpropionic acid was a major urinary metabolite. It was found primarily in the 24-hour urines (free fraction) although it was also present in the 48-hour urines, where it was the only metabolite detected. Caffeic and ferulic acids were also detected as major metabolites in the 24-hour urines (free fraction) as was, to a lesser extent, hydrocaffeic acid. *m*-Coumaric acid was not observed on the chromatograms. The results were qualitatively similar when a dose of 500 mg/kg was given.

The above 24-hour urines (bound fraction) contained caffeic and ferulic acids as the major metabolites but relatively small amounts of *m*-hydroxyphenylpropionic acid. Another compound was detected which corresponded to 4-vinylcatechol both in Rf values and colour reaction. It was present only in trace amounts after dosing with 100 mg/kg, but readily observed on the chromatograms when caffeic acid was given in a dose of 500 mg/kg. With higher doses another metabolite was observed which corresponded to 4-ethylcatechol. Both of these metabolites were detected in four of the five rats given the higher doses. Further confirmation of the nature of these metabolites was obtained by removing the acidic compounds by extraction with 5 / $NaHCO_3$ and chromatographing the remaining compounds on a thin-layer cellulose plate with 20 % aqueous potassium chloride as solvent. The 4-vinylcatechol and 4-ethyl-

catechol areas were scraped off and eluated with water The former showed an absorption maximum at 257–258 mμ and a fluorescence maximum at 343 mμ which are in agreement with those for 4-vinylcatechol (table 3) In the case of 4-ethylcatechol confirmation was more difficult and it appears that this metabolite was present only in very small amounts. The eluates from the 4-ethylcatechol areas gave weak absorption at approx. 275–280 mμ but their fluorescence maxima at 321–322 mμ is indicative of 4-ethylcatechol. None of the aforementioned metabolites was detected in control urines (four rats).

When caffeic acid (100 mg/kg) was given by intraperitoneal injection to three rats fed the purified diet, the 24-hour urines (free fraction) also contained *m*-hydroxyphenylpropionic acid, although the amounts present were considerably less than those found after oral dosage. The most prominent metabolites were caffeic and ferulic acids. When the common bile duct was tied and severed (five rats), intraperitoneal administration of caffeic acid (100 mg/kg) resulted in the excretion of caffeic, ferulic and to a lesser extent, hydrocaffeic acids but *m*-hydroxyphenylpropionic acid could not be detected in any of the 24-hour urines.

The biliary excretion of caffeic acid and its metabolites was studied in five rats after intraperitoneal administration (100 mg/kg) The initial experiment showed that the free fraction of the 24-hour bile did not contain any of these compounds and subsequently this step was omitted Ferulic acid was detected in all five 24-hour bile samples following incubation with β-glucuronidase and in two cases, caffeic acid was found. No other caffeic acid metabolites were observed in these samples.

The finding that ferulic acid is the main biliary metabolite of caffeic acid necessitated the study of its metabolism and excretion When ferulic acid (100 mg/kg) was administered intraperitoneally it was rapidly excreted in the bile. Chromatograms of extracts of the 0–5-hour bile after β-glucuronidase treatment showed prominent ferulic acid spots. These were not detected in the 5–24-hour bile. The urinary metabolites (free fraction) of ferulic acid (100 mg/kg) were found to be the same either after oral or intraperitoneal administration. The most prominent compound on the chromatograms was ferulic acid. *m*-Hydroxyphenylpropionic acid was readily detected and in addition smaller amounts of hydroferulic acid. No hydrocaffeic acid was seen on the chromatograms and no or at most, a trace of caffeic acid was found.

Discussion

The findings described above demonstrate that the intestinal microflora has the ability to effect a number of metabolic reactions with phenolic phenylacetic, phenylpropionic and cinnamic acids. Of the four re

actions encountered (reduction of a double bond, dehydroxylation, decarboxylation and demethylation) the one which has hitherto been most widely studied is dehydroxylation (SMITH 1966) The present results thus confirm previous reports of the ability of the intestinal microflora to remove a *p*-hydroxyl group from homoprotocatechuic and caffeic acids (BOOTH & WILLIAMS 1963a & b SCHELINE 1967 PEREZ-SILVA *et al* 1966). The former compound has been shown to undergo *m*-dehydroxylation to a minor extent when administered orally to rabbits (SCHELINE *et al* 1960). This reaction could not be studied in the present investigation because small amounts of 4-hydroxyphenylacetic acid were normally detected in the caecal incubates.

BOOTH & WILLIAMS (1963a) reported that intestinal microorganisms converted caffeic acid to both *m*-coumaric and *m*-hydroxyphenylpropionic acids. The former metabolite was not observed in any of the present experiments with caffeic acid. This finding seems to indicate that dehydroxylation occurred following reduction to hydrocaffeic acid although the possibility exists that the capacity of the caecal microorganisms to reduce a double bond greatly exceeds their capacity to bring about dehydroxylation. Thus, *m*-coumaric acid would be reduced rapidly on its formation and would thus not be detected. On the other hand, *m*-coumaric acid reduction was incomplete when this compound was used as a test substance. This finding supports the assumption that in the present experiments caffeic acid was first reduced before being dehydroxylated.

Decarboxylation by the intestinal microorganisms has been studied mainly from the point of view of the metabolism of amino acids which can be converted to amines by many intestinal bacteria (MELNYKOWYCZ & JOHANSSON 1955) ROCHE *et al* (1962) obtained evidence which indicated that triiodothyronine when administered to rats was decarboxylated to triiodothyronamine in the intestine. Protocatechuic acid was decarboxylated to catechol when incubated with rat faecal and caecal extracts (BOOTH & WILLIAMS 1963b). Previous reports from this laboratory (SCHELINE 1966a & b & 1967) and the present study show that intestinal decarboxylation is an important reaction for many phenolic acids. Because these substances occur widely in plants and are therefore normal dietary constituents, it seems reasonable to assume that many phenols resulting from phenolic acid decarboxylation can be expected to be found in the urine, provided that suitably sensitive methods are used for their detection.

FINKLE *et al.* (1962) have reported that several strains of the genus *Aerobacter* were able to decarboxylate 4-hydroxycinnamic acids to vinyl derivatives. The phenylacetic and phenylpropionic acid derivatives were

however not decarboxylated The capacity of the caecal contents to decarboxylate several classes of phenolic acids could be explained by the presence of a microorganism containing a relatively nonspecific decarboxylase or by the presence of several different decarboxylases found in one or more microorganisms. The finding that treatment of the caecal extract at 80 for 15 minutes before incubation in order to destroy vegetative forms, abolished decarboxylation of the phenolic benzoic and phenylacetic acids, but not the cinnamic acids, indicates that more than one microorganism and decarboxylase is involved. Based on the substrate specificity and the products formed the caecal 4-hydroxycinnamate decarboxylase is similar to that found in *Aerobacter* although the heat resistance of the caecal microorganisms suggest that a different genus is responsible.

The reduction of double bonds is a characteristic reaction carried out by microorganisms. It is well known that unsaturated fatty acids are hydrogenated by the microorganisms of the rumen and lower intestine (WILDE & DAWSON 1966) It has also been shown that castor oil, which contains ricinoleic acid, gives rise to hydroxystearic acid in the faeces when fed to rats or man (WATSON 1965) There is evidence that the intestine is the site of this conversion although the reaction has not been demonstrated in pure or mixed intestinal bacterial cultures.

The present results show that reduction of the phenolic cinnamic acids by caecal microflora is a major reaction of these compounds and thus confirm the report of BOOTH & WILLIAMS (1963a) that rat caecal contents reduce caffeic acid to hydrocaffeic acid. It is known that rats, rabbits and man are capable of reducing caffeic and ferulic acids to their hydro derivatives (BOOTH *et al* 1957) The finding that caffeic acid, when injected intraperitoneally into rats which have previously had their common bile ducts tied off and severed in order to prevent biliary excretion, is partly metabolized to hydrocaffeic acid, demonstrates that the reduction also takes place in the body Therefore, we do not know to what extent the intestinal microflora are involved in the reduction of the phenolic cinnamic acids fed to animals.

Many examples of cleavage of aromatic ethers in the body are known and it has been found that this reaction is one of the oxidative reactions of the liver microsomal enzymes. MCMAHON *et al* (1963) studied the metabolism of a series of *p*-nitrophenyl ethers both by rat liver microsomes and *in vivo* and they found good correlation between the rates of dealkylation.

However other reports in the literature have described cases of demethylation which could most easily be explained on the basis of an intestinal reaction. An example of this is the finding that the flavonoid hesperi-

din is metabolized in the rabbit to several hydroxy acids including 3,4-dihydroxyphenylpropionic and *m*-hydroxyphenylpropionic acids (BOOTH *et al.* 1958). Formation of the latter compound requires ring fission, demethylation and dehydroxylation and it seems likely that the entire sequence of steps occurs in the intestine. As the final step is one which is carried out by the intestinal microorganisms, other explanations would require a rather involved interplay between metabolism in the body, biliary excretion of large amounts of 3,4-dihydroxyphenylpropionic acid and intestinal reactions. However, 3,4-dihydroxyphenyl compounds are probably mainly excreted in the bile not as such but as conjugates of their methyl derivatives. This has been found in the present study with caffeic acid which is mainly excreted in the bile as the glucuronide of ferulic acid. Moreover, DACRE & WILLIAMS (1962) reported that protocatechuic acid is excreted in the bile largely as the glucuronide of vanillic acid. The present finding that hydroisoferulic acid is metabolized to *m*-hydroxyphenylpropionic acid by caecal microorganisms, further supports the view that the above mentioned metabolic reactions of hesperidin occur in the intestine. The results of the present study together with the previous findings that demethylation also occurred with several phenolic benzoic acid derivatives (SCHELINE 1966b) indicate that the influence of the intestinal flora must be taken into account when dealing with the metabolism of ethers.

The metabolism of caffeic acid in rats and humans has been studied in detail by BOOTH *et al.* (1957). They found that ingestion of this compound led to the excretion of more than ten phenolic acids in the urine. In the rat most of the metabolites contained the 3-carbon side chain and the major metabolite was *m*-hydroxyphenylpropionic acid. These metabolites were also detected in the urine following intraperitoneal injection of caffeic acid and this was assumed to indicate that the intestinal microorganisms do not play a role in its metabolism. Subsequently, however, BOOTH & WILLIAMS (1963a & b) were able to demonstrate that the intestinal contents of many species were capable of dehydroxylating a number of phenolic compounds including caffeic acid which was converted to *m*-coumaric acid and *m*-hydroxyphenylpropionic acid.

As the evidence now available indicates that dehydroxylation is a reaction which occurs only in the intestine of animals and not in other tissues (SHAW *et al.* 1963, SMITH 1966), the presence of dehydroxylated metabolites of caffeic acid in the urine following intraperitoneal dosage might best be explained by biliary excretion of caffeic acid, or of a metabolite which is capable of being metabolized to *m*-hydroxyphenylpropionic acid by the intestinal flora. The present results show that *m*-hydroxyphenylpropionic acid is a major urinary metabolite of caffeic

acid in rats following oral administration and that it is also excreted following intraperitoneal injection, although to a lesser extent. However in the latter case, the formation of *m*-hydroxyphenylpropionic acid is dependent on the excretion of ferulic acid, and to a limited extent on caffeic acid, itself, as a conjugate into the bile. This conjugate would be expected to be very poorly absorbed from the intestine and instead be attacked by bacterial enzymes there, i.e first by β-glucuronidase (SMITH & WILLIAMS 1966). Subsequently the aglycone could be metabolized to *m*-hydroxyphenylpropionic acid as found in the present experiments. No dehydroxylated metabolite was detected in the urine when caffeic acid was given intraperitoneally to rats prevented from excreting bile into the intestine.

The finding that caffeic acid was decarboxylated by caecal microorganisms to 4-vinylcatechol and 4-ethylcatechol revealed a new pathway in its metabolism. These metabolites were detected in the urine of rats given caffeic acid although they were not major metabolites. It is obvious that the results obtained initially from the experiments with caecal microorganisms in this case were of considerable value in studying caffeic acid metabolism in rats. We suggest that increased awareness of the metabolic capabilities of the intestinal microflora is warranted and that studies of the metabolism of drugs and foreign compounds should more fully consider this possibility

Summary

The metabolism of a number of phenolic phenylacetic, phenylpropionic and cinnamic acids by the rat intestinal microflora has been studied. Reduction of the double bond in *o*-coumaric, *m*-coumaric, *p*-coumaric, caffeic, isoferulic and ferulic acids was observed. Dehydroxylation of homoprotocatechuic and hydrocaffeic acids to the corresponding *m*-hydroxy acids was found. These *m*-hydroxy acids were also formed from phenolic acids capable of being metabolized to homoprotocatechuic or hydrocaffeic acids. Decarboxylation of phenylacetic and cinnamic acids containing a free *p*-hydroxyl group was observed. This reaction did not take place with the corresponding phenylpropionic acids. Demethylation occurred with the 3-hydroxy-4-methoxy and 4-hydroxy-3-methoxy- derivatives of phenylacetic, phenylpropionic and cinnamic acids.

The significance of the metabolism by the intestinal microflora was studied by administering caffeic acid to rats. *m*-Hydroxyphenylpropionic acid was a major urinary metabolite and was shown to have an intestinal origin. The decarboxylated metabolites, 4-vinylcatechol and 4-ethylcatechol were detected in the urine as conjugates.

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Studies on the Tachyphylaxis to α Methyl Metatyramine with Special Reference to its Central Stimulating Action

By

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(Received September 2, 1967)

A central stimulating effect of α methyl metatyramine in addition to its peripheral sympathomimetic activity has previously been reported. A central and peripheral catecholamine-depleting action of this drug has also been described. (PORTER, TOTARO & LEIBY 1961 GYÖRGY MOLNÁR & DODÁ 1963 PFEIFFER & GALAMBOS 1967). We have confirmed this and also found that *tachyphylaxis to both central and peripheral actions* develop after repeated injections of the drug. This is in contrast to amphetamine, which is capable of maintaining central stimulation for a long time. Another difference between the two drugs was found In doses necessary to produce stimulation α methyl metatyramine caused significant depletion of catecholamines (CA) both centrally and peripherally whereas amphetamine produces such an effect only after excessive doses (CARLSSON *et al.* 1965 1966a & b CARLSSON 1967) These observations suggested a difference in the mode of action of the two drugs.

In the present paper an attempt has been made to elucidate the mode *of action of α methyl metatyramine by studying amine levels and responses* to certain drugs after development of tachyphylaxis. The results suggest *that this amine acts by releasing CA stores. Tachyphylaxis appears to be* due to depletion of these stores.

Materials and Methods

The experiments were performed on female albino mice (N M R.I strain) weighing 18–22 g. Amine analyses were performed o six pooled brains, noradrenaline (NA) according to BERTLER, C RLSSON & ROSENGREN (1957) and dopamine (DA) by the method of CARLSSON & WALDECK (1958) modified by CARLSSON & LINDQVIST (1962). Gross behaviour especially signs of central stimulation and peripheral sympathomimetic effects, are studied after various kinds of drug treatment (see under Results).

Results

α-Methyl metatyramine was injected intraperitoneally into mice. The animals received 4 injections of the amine at intervals of 8 hours 12.5 25 50 100 mg/kg. A small test dose, 12.5 mg/kg, was given 4 hours after the last mentioned injection, and one hour later the mice were killed by decapitation. Amine analyses were performed on the brains. After the first dose the animals showed piloerection exophthalmus, salivation, increased motility and aggressiveness. These effects tended to be progressively weaker after the subsequent doses, in spite of the increase in dose. After the final test dose, which was equal to the initial dose, the response was considerably weaker than initially The tachyphylaxis thus developed was accompanied by around 80 / depletion of NA and around 65 / depletion of DA in the brain (table 1) Other groups of mice were given protriptyline 20 mg/kg intraperitoneally 30 min. before each injection of α-methyl metatyramine. The pharmacological effects of the amine, when given in the smallest dose, were largely blocked by protriptyline pretreatment. The effects of higher doses were less efficiently blocked. The effect on brain NA was similarly prevented whereas the effect on DA was if anything entranced (table 1).

The ability of a few other drugs to modify the tachyphylaxis was also studied.

Mice in a group of five made tachyphylactic to α methyl metatyramine as described above were given α methyldopa intraperitoneally 400 mg/kg 2 hours after the 100 mg/kg dose of α methyl metatyramine. Eight hours later the usual test dose of α-methyl metatyramine was given Central stimulation was even weaker than in a control group of five tachyphylactic animals which had received no α-methyldopa. Peripheral effects were about the same in the two groups. The experiment was performed both at room temperature (24°) and at 30 with essentially the same result.

In another experiment a group of five normal mice were given an i.p. injection of α-methyldopa 400 mg/kg. After 12 hours, when the NA level in brain could be expected to be reduced by at least 75 per cent and the DA level by about 30 / (CARLSSON & LINDQVIST 1962), the usual test dose of α methyl metatyramine was given The animals were very excited and showed signs of strong sympathetic stimulation as compared with animals made tachyphylactic to α methyl metatyramine as described above. The experiment was repeated twice with esentially the same result.

The following experiment was performed with (+)-amphetamine. One group of five tachyphylactic mice received the test dose of α-methyl metatyramine. The animals were observed for about 15 minutes to make sure that tachyphylaxis had developed and then (+)-amphetamine 5 mg/kg

Table I

Effects of repeated doses of α-methyl metatyramine with and without protriptyline pretreatment on brain catecholamine levels.

One group of animals received 4 injections of the amine at intervals of 8 hours: 12.5, 25 50, 100 mg/kg. A small test dose of 12.5 mg/kg was given 4 hours after the last mentioned injection, and the animals were killed one hour later Another group of mice was given protriptyline 20 mg/kg 30 min. before each injection f α-methyl metatyramine. All injections were given i.p. (The figures refer to the base).

Normal values are Noradrenaline 0.41 ± 0.012 μg/g (14 analyses) and dopamine 0.75 ± 0.029 μg/g (11 analyses).

Noradrenaline μg/g tissue		Dopamine μg/g tissue	
α-methyl metatyramine	protriptyline + α methyl metatyramine	α-methyl metatyramine	protriptyline + α-methyl metatyramine
0.09	–	0.16	–
0.07	–	0.22	–
0.10	0.22	0.29	0.24
0.06	0.23	0.31	0.24
0.06	0.18	0.31	0.23

Data given on one and the same line refer to one experiment, in which the animals were treated in parallel with α-methyl metatyramine and protriptyline and α methyl metatyramine.

was given intraperitoneally One control group of five mice was given the test dose but no (+)-amphetamine, and a group of five non tachyphylactic animals were given only (+)-amphetamine In the first mentioned group of tachyphylactic mice the injection of (+)-amphetamine was followed by a conspicuous central stimulation of about the same degree as in the non-tachyphylactic animals. With regard to peripheral sympathomimetic effects, however the response to (+)-amphetamine seemed to be somewhat weaker in the first-mentioned group The experiment was repeated twice with essentially the same result.

The ability of certain drugs to interfere with the response to a single dose of α-methyl metatyramine was investigated as follows. The experiments were performed on mice in groups of five. They were all repeated with essentially the same results.

Nialamide 100 mg/kg was injected intraperitoneally and one hour later α methyl metatyramine 12.5 mg/kg was given intraperitoneally No potentiation of central or peripheral effects of the amine was observed.

Six hours after administration of reserpine 10 mg/kg i.p α-methyl metatyramine 50 mg/kg was injected i p The actions of the amine were almost completely blocked by reserpine.

Phenoxybenzamine (bensylytum NFN), 20 mg/kg injected i p 45 min. before the administration of α-methyl metatyramine 12.5 mg/kg, was found to block both central and peripheral actions of the test dose.

The i.p injection of propranolol 10 mg/kg 45 min before the test dose of α-methyl metatyramine caused little or no modification of the response.

Discussion

The development of tachyphylaxis to α methyl metatyramine was accompanied by a marked depletion of catecholamines in the brain That this amine is acting indirectly i.e. by release of endogenous amines, is also indicated by the fact that its actions are largely blocked by reserpine and protriptyline. The data with protriptyline are particularly illuminating, since this agent was found to block the pharmacological actions and NA depletion simultaneously Protriptyline is known to block an amine concentrating mechanism located at the level of the cell membrane of peripheral and central NA neurones, whereas amine uptake by central DA neurones is resistant to this drug (CARLSSON *et al.* 1966b HAMBERGER 1967). Accordingly it was found that depletion of DA, in contrast to NA, could not be blocked by protriptyline There was even a tendency to an enhanced DA depletion after protriptyline. The mechanism of this enhanced effect is not clear Possibly protriptyline inhibits metabolic conversion of α-methyl metatyramine (cf SULSER *et al* 1966) Such an effect would probably lead to an underestimation of the blocking action on NA depletion.

Unpublished data from this laboratory show that the ability of α methyl metatyramine to deplete 5-hydroxytryptamine stores is slight.It may therefore be concluded that the central actions of this agent are mainly due to release of catecholamines Since protriptyline did not block DA depletion, although the pharmacological effects were largely prevented, this catecholamine seems to play a less important role than NA in producing these actions. In support of this assumption, α-methyl meta tyramine was found to cause a more pronounced depletion of NA than of DA.

In view of these findings and considerations it seems justifiable to conclude that the tachyphylaxis observed after α methyl metatyramine treat ment is largely due to depletion of amines – chiefly NA – in the central and peripheral nervous system.

α-Methyldopa given to tachyphylactic mice restored the effect of a test dose of α-methyl metatyramine peripherally but not centrally where the effect was even weakened. This suggests that α methyl noradrenaline,

formed from the injected α methyldopa, cannot serve as a functional substitute for NA in mouse brain However when α methyldopa was injected into normal mice, a subsequent test dose of α methyl metatyramine produced marked central stimulation and peripheral sympathomimetic actions. This points in the opposite direction i.e. that the α methyl NA replacing the NA is functionally active. The weak central effect of a test dose on tachyphylactic mice given α-methyldopa might be explained on the basis of diminished accumulation of α methyl noradrenaline in the granules in the presence of metaraminol (cf ANDÉN 1964) In order to solve this question the amines involved must be measured quantitatively in the brain.

There was no central cross-tachyphylaxis between α methyl metatyramine and (+)-amphetamine, suggesting that the two drugs have a different mode of action. HANSON (1966) has shown that the presence of small amounts of NA is necessary for amphetamine to produce its central stimulating effect. Amphetamine appears to act by releasing a small extragranular pool without any depletion of the granules, unless excessive doses are used α Methyl metatyramine on the other hand probably produces its effects by releasing NA from the stores, i e the granules, resulting in a significant depletion. It may be assumed that the first mentioned extragranular pool is not depleted in animals made tachyphylactic to α methyl metatyramine.

Inhibition of monoamine oxidase did not result in potentiation of the actions of α methyl metatyramine. This was unexpected since some of the NA released from the granules might be assumed to undergo deamination through intraneuronal monoamine oxidase. The potentiation thus obtained is possibly counterbalanced by the competitive action of NA accumulating in the cytoplasm after inhibition of the enzyme thus preventing α-methyl metatyramine from gaining access to granular binding sites.

The blockade of the effects of α-methyl metatyramine by phenoxybenzamine suggests that the NA released by its analogue may act on receptors of the α type in the brain. The negative results obtained in the present study with a β-blocking agent do not exclude the possibility that β-adrenergic receptors also play a role in the brain

Summary

After repeated injections of α methyl metatyramine into mice, tachyphylaxis of both central stimulating and peripheral sympathomimetic actions of the drug was observed This was accompanied by depletion of brain catecholamines. Experiments with protriptyline and reserpine

indicate that α-methyl metatyramine acts indirectly *via* release of monoamines among which noradrenaline appears to play a dominating role. Tachyphylaxis thus seems to be due to monoamine depletion. As indicated *i.a.* by absence of crosstachyphylaxis, the central stimulating actions of α-methyl metatyramine and (+)-amphetamine are caused by different mechanisms.

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Potentiation of 5-Hydroxytryptamine Release from Platelets by Desmethylation of Chlorpromazine and Related Agents

By

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(Received December 5, 1967)

Chlorpromazine and some related agents produce 5-hydroxytryptamine (5HT) depletion from blood platelets *in vitro* (BARTHOLINI *et al* 1961 PAASONEN 1964) They also induce haemolysis of red cells (CHAPLIN *et al* 1952 AHTEE & PAASONEN 1965). There are structure-dependent differences in the capacity of phenothiazine derivatives and related compounds to liberate 5HT from platelets and cause haemolysis (AHTEE & PAASONEN 1965 AHTEE 1966a). It has been shown, for instance, that desmonomethylation of the terminal methyl amine group in the side chain attached to N_{10} of chlorpromazine imipramine and amitriptyline increases the 5HT releasing capacity of these compounds (AHTEE & PAASONEN 1966b AHTEE 1966a). The present work was undertaken in order to study further the mechanism of this potentiation.

Methods

Under ether anaesthesia male albino rabbits weighing 2 5 to 3.4 kg were bled from carotid artery by means of a polyethylene cannula. The blood was mixed with [illegible] vol. of 1.5 % disodium edetate (EDTA) in 0.7 % sodium chloride. The platelet-rich plasma was obtained by centrifugation of the blood at about 130 × g for 10 to 20 min. at room temperature. It contained 800,000 to 1 200,000 platelets per mm³ As value for platelet volume, 1 µl/10⁸ platelets was used. Platelet count were made with the phase contrast microscope. Duplicate samples of platelet-rich plasma or of whole blood were incubated for 30 to 180 min in air atmosphere with or without dditional treatment by gentle shaking at 37 All the experiments were carried out in polyethylene or polypropylene vessels and polypropylene pipettes were used Into each ml f sample was added 0.1 ml or less of the drug solution or solvent only

After incubation the platelets from 1 t 2 ml of plasma were separated by centrif gation at about 4000 g for 20 min. at a temperature below 5 The 5HT from platelets was

estimated spectrophotofluorometrically according to WEISSBACH *et al.* (1958) as modified by CROSTI & LUCCHELLI (1962).

In some experiments the extent of haemolysis was estimated colorimetrically (AHTEE 1966a).

The drugs were kindly supplied by the following pharmaceutical companies: Chlorpromazine hydrochloride (May & Baker Ltd., Dagenham), desmonomethylchlorpromazine maleate (Rhône-Poulenc, Paris), desdimethylchlorpromazine maleate (May & Baker Ltd., Dagenham), imipramine hydrochloride (Orion Oy, Helsinki), desipramine hydrochloride (J. R. Geigy A.G., Basel), desdimethylimipramine as base (Lakeside Laboratories, Inc., Milwaukee, Wisconsin), amitriptyline hydrochloride and nortriptyline hydrochloride (H. Lundbeck & Co. A/S, Copenhagen).

Desmonomethylchlorpromazine, desdimethylchlorpromazine and desdimethylimipramine were dissolved in 0.5 ml of 0.1 N HCl and further diluted with saline. The other substances were dissolved in saline. In the final concentrations used hydrochloric acid had no platelet 5HT releasing effects.

Chlorpromazine and its demethylated derivatives were extracted from alkaline homogenates by the procedure described by SALZMAN & BRODIE (1956) and determined spectrophotometrically. The wave lengths for the estimation of chlorpromazine were 255 and 270 mμ, and for desmethyl derivatives, 255 mμ.

Heptane-buffer and petroleum ether-buffer partition coefficients were determined by shaking 10^{-4} M phenothiazine solution in 0.1 M phosphate buffer pH 7.4 with heptane or petroleum ether at room temperature for 1 hr. The phenothiazine concentrations were then measured from the buffer, heptane and petroleum ether.

Student's t-test was used for the statistical analysis of the results.

Results

5HT release from platelets

Table 1 shows the release of 5HT from rabbit platelets after incubation for 3 hr of platelet rich plasma with 3×10^{-5} M, 10^{-4} M and 3×10^{-4} M of chlorpromazine, desmonomethylchlorpromazine, desdimethylchlorpromazine, imipramine, desimipramine, desdimethylimipramine, amitriptyline and nortriptyline. The values presented express the amount of liberated 5HT as % of the 5HT content of the corresponding control samples incubated for 3 hr.

Amitriptyline was slightly more active than chlorpromazine or imipramine. Demethylation of the tertiary dimethylamine group to a secondary monomethylamine group increased the potency (desmonomethylchlorpromazine, desipramine, nortriptyline). Among these the desmonomethylated derivatives of nortriptyline were slightly more active than the two other compounds. The effect of desmonomethylation on the 5HT releasing capacity of the compounds studied was most pronounced when the platelet-rich plasma was incubated with 10^{-4} M concentration. At these concentrations the differences between the di- and monomethyl derivatives were highly significant ($P < 0.001$).

Table 1

The amount of 5HT released (in % of the control value) from platelets during 3-hr incubation of platelet-rich plasma with three concentrations of various compounds at 37

Compound	5HT release % ± s.e.m. (N)					
	3×10^{-5} M		10^{-4} M		3×10^{-4} M	
Chlorpromazine.	3 ± 2	(5)	16 ± 4	(5)	84 ± 3	(4)
Desmonomethyl-chlorpromazine	9 ±	(4)	60 ± 8	(5)	91 ± 3	(4)
Desdimethyl-chlorpromazine	8 (4 11)	(2)	42 (15 69)	(2)	9? (92, 92)	(2)
Imipramine	0 ± 3	(8)	19 ± 4	(8)	71 ± 4	(7)
Desmonomethyl-imipramine	10 ± 4	(8)	57 ± 10	(7)	90 ± 2	(8)
Desdimethyl-imipramine	0 ± 7	(4)	53 ± 16	(4)	87 ± 4	(4)
Amitriptyline	17 ± 4	(3)	30 ± 3	(3)	86 ± 4	(3)
Nortriptyline.	17 ± 2	(3)	70 ± 8	(4)	95 ± 1	(3)

) Due to the lack of the substance no more experiments could be done.
The corresponding mean values for chlorpromazine were 10, 33 and 80 %, respectively

Both chlorpromazine and its desmonomethyl derivative released more 5HT the longer the platelet rich plasma was incubated with these derivatives (fig. 1). At a concentration of 1 5 × 10^{-4} M desmonomethylchlorpromazine released slightly more 5HT than a 3 × 10^{-4} M concentration of chlorpromazine. On the other hand a 10^{-4} M concentration of desmonomethylchlorpromazine released less 5HT than a 2 × 10^{-4} M concentration of chlorpromazine.

Desdimethylchlorpromazine probably released more 5HT than chlorpromazine but less than desmonomethylchlorpromazine (table 1) The activity of desdimethylimipramine in this respect was equal to that of desipramine.

Accumulation of chlorpromazine and its derivatives in platelets and red cells

The absorption of chlorpromazine and its desmethyl derivatives into platelets was measured after 1-hr incubation of whole blood with 5 × 10^{-5} M of these agents (fig. 2). During this time platelets took up 1 06 mg/ml

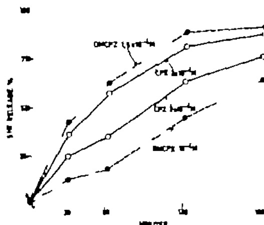


Fig. 1 The mean release of 5HT from platelets during incubation f platelet-rich plasma with various concentrations of chlorpromazine (CPZ) and desmonomethylchlorpromazine (DMCPZ) at 37 (3 experiments)

(platelet substance) of chlorpromazine, 1 66 mg/ml of desmonomethylchlorpromazine and 2.12 mg/ml of desdimethylchlorpromazine. On the other hand, no such differences were found in the accumulation of these derivatives in the red cells and the corresponding values were 0 13 0 13 and 0 19 mg/ml, respectively

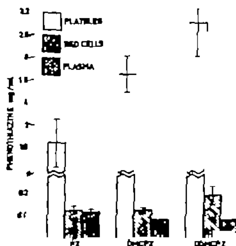


Fig. 2. The mean amounts ± s.e.m. of chlorpromazine (CPZ), desmonomethylchlorpromazine (DMCPZ) and desdimethylchlorpromazine (DDMCPZ) in platelets, red cells and plasma after 1-hr incubation of whole blood with 5×10^{-5} M concentrations of these compounds at 37 N = 3 to 5.

Table 2

The partition coefficients of chlorpromazine (CPZ), desmonomethylchlorpromazine (DMCPZ) and desdimethylchlorpromazine (DDMCPZ) between heptane or petroleum ether and 0.1 M phosphate buffer pH 7.4. Compounds were added in the buffer at a concentration of 10^{-4} M and shaken for 1 hr at room temperature.

Volume ratio: Heptane or petroleum Ether/Buffer	Partition coefficients					
	Heptane/Buffer			Petroleum ether/Buffer		
	CPZ	DMCPZ	DDCPZ	CPZ	DMCPZ	DDCPZ
1/5	77	3.0	0.7[illegible]	62	2.3	0.76
1/10	56	1.3	0.34	5[illegible]	1.1	0.34
1/50	13	0.2	0.05	17	0.2	0.05

Haemolysis of red cells

During incubation for 1 hr at concentrations of 10^{-4} M, 3×10^{-4} M and 10^{-3} M desdimethylchlorpromazine caused haemolysis of 0.4 %, 2 % and 72 % of red cells, respectively. The corresponding values for chlorpromazine were 4 %, 17 % and 89 %. The desmethyl derivatives of imipramine also caused less haemolysis than the parent compound, the percentage values (means from two experiments with duplicate samples) after 1 hr incubation at the above concentrations were imipramine 0 %, 2 % and 43 %, desipramine 0 %, 1 % and 11 %, desdimethylimipramine 0 %, 0 % and 2 %, respectively.

Solubilities of phenothiazines

Table 2 represents the partition coefficients of chlorpromazine and its desmethyl derivatives between heptane or petroleum ether and 0.1 M phosphate buffer pH 7.4. The figures are means of 2 experiments which gave values close to each other. Chlorpromazine had a higher affinity for these fat solvents than desmonomethylchlorpromazine. Further demethylation of the terminal amine group to desdimethylchlorpromazine decreased the solubility in fat solvents even more.

Discussion

The present results show that desmonomethylation of the terminal dimethylamine group in the side chain of chlorpromazine, imipramine and amitriptyline increases the capacity of these compounds to release

5HT from platelets *in vitro* Desdimethylation does not further increase this effect. The increased activity cannot simply be due to potentiation of the membrane effects, as desmonomethylation reduced the capacity to cause haemolysis of red cells in the compounds studied (AHTEE 1966a). The desdimethyl derivatives of chlorpromazine and imipramine were also much weaker haemolyzing agents than chlorpromazine and imipramine.

The accumulation of chlorpromazine, its sulphoxide and desmonomethyl derivative, as well as N hydroxyethylpromethazine in platelets and and red cells parallels the platelet 5HT releasing capacities and haemolytic actions of these compounds (AHTEE & PAASONEN 1966a AHTEE 1966a). However though platelets accumulate more desdimethylchlorpromazine than desmonomethylchlorpromazine, the former is not a more potent 5HT releaser Similarly desdimethylchlorpromazine accumulates slightly more in the red cells than chlorpromazine, but causes less haemolysis

Studies in progress (SOLATUNTURI & AHTEE) on the intracellular distribution of phenothiazines indicate that chlorpromazine and desmonoethylchlorpromazine become concentrated in the 5HT containing fractions to a similar extent, i.e. about 30 %. The corresponding value for chlorpromazine sulphoxide was about 10 %, i.e. most of the chlorpromazine sulphoxide remained in the supernatant and only 10 % in the 5HT fractions. Since chlorpromazine sulphoxide also releases 5HT from platelets (AHTEE & PAASONEN 1965 AHTEE 1966a) the intracellular distribution of phenothiazines seems to bear no clear relationship to their 5HT releasing capacity

YATES *et al* (1964) have shown that demethylated derivatives of imipramine and amitriptyline inhibit the uptake 5HT by platelets less than do their parent compounds. It is unlikely therefore that the more potent 5HT liberation by the desmonomethylated compounds are due to an increased inhibition of the re-uptake of the liberated amine.

With regard to their physicochemical properties, the secondary amines are known to differ from the tertiary amines in many ways. For instance, the pK_a value for imipramine was 9 5 and that for desipramine 10.2 (GREEN 1967) The corresponding figures for amitriptyline and nortriptyline were 9.3 and 9 8 respectively (ELFVING & KENTTÄMAA, personal communication) This increase in pK_a value is in accordance with the finding that the partition coefficient between heptane or petroleum ether and phosphate buffer pH 7 4 decreases with desmethylation. Similar results were found by KOHL *et al* (1964) using chlorpromazine derivatives. These results suggests that lipid solubility decreases by des- and, even more, by didesmethylation. One would expect the decreased lipid solubility to bring about decreased membrane effects. Hence these findings are more

in accordance with the diminished haemolytic capacity than with the increased 5HT releasing activity of the compounds. Surface activity is another physicochemical property which might be responsible for the membrane effects of the compounds studied. This, however was not modified by desmonomethylation (AHTEE 1966b)

In this connection it should also be mentioned that when injected intravenously into rabbits desmonomethylchlorpromazine was a more active 5HT liberator from the lungs and duodenum than chlorpromazine (AHTEE & PAASONEN 1967). In addition more desmonomethylchlorpromazine was concentrated in these tissues than chlorpromazine These findings indicate that the results obtained by using platelets can be applied to other tissues (PAASONEN 1965) In fact, an interesting result of this work is the finding that desmonomethylchlorpromazine releases 4 to 6 times as much adrenaline and noradrenaline from the perfused adrenal gland than chlorpromazine (VAPAATALO *et al* 1966).

Our results suggest that the secondary demethylated derivatives of the compounds studied exert greater effects on the cellular monoamine metabolism than the tertiary dimethylamine containing compounds. This is in accordance with the suggestion that the demethylated derivatives are the main metabolites and perhaps the active agents responsible for the antidepressant effects of the substituted phenothiazines and related compounds ((SULSER *et al* 1962 BICKEL *et al.* 1967)

Summary

The release of 5-hydroxytryptamine (5HT) from rabbit blood platelets by chlorpromazine, imipramine, amitriptyline and their demethylated derivatives was studied *in vitro* The desmonomethylated derivatives, desmonomethylchlorpromazine, desipramine and nortriptyline, decreased significantly more platelet 5HT than the corresponding parent compounds did Desdimethylchlorpromazine liberated as much 5HT as, or slightly more than chlorpromazine, and desdimethylimipramine in this respect was equal to desipramine.

Platelets accumulated about twice as much desmonomethylchlorpromazine and desdimethylchlorpromazine as chlorpromazine The uptake of desdimethylchlorpromazine was slightly higher than that of desmonomethylchlorpromazine. There were no such clear differences in the accumulation of these derivatives in the red cells.

The 5HT releasing activity of the compounds did not run parallel with their haemolytic effect which decreased after progressive demethylation. The solubility of chlorpromazine into heptane or petroleum ether was

higher than that of desmonomethylchlorpromazine. Further demethylation decreased the solubility still more. The physicochemical properties studied do not explain why desmonomethylation potentiates the 5HT releasing capacity of the compounds studied.

Acknowledgements

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Determination of 5-(p-hydroxyphenyl)-5-phenyl-hydantoin (HPPH) in Urine by Thin-layer Chromatography

By

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(Received November 6, 1967)

BUTLER (1957) and MAYNERT (1960) have shown that following oral or intravenous administration in man phenytoin (diphenylhydantoin) is mainly excreted as 5-(p-hydroxyphenyl)-5-phenyl hydantoin (HPPH) in the urine. Moreover MAYNERT showed what BUTLER did not succeed in demonstrating, that hydroxyphenytoin (abbreviated to HPPH in the following) is coupled to glucuronic acid. It can be released from this either by enzyme action or by hydrolysis with strong acids.

Following hydrolysis, BUTLER used a countercurrent distribution apparatus for the isolation and quantitative determination of HPPH in urine. The concentration was calculated from the difference between the absorption of the substance at 250 and 260 nm, respectively The accuracy of the method of analysis was not stated. MAYNERT used paper chromatography on which after development, the chromatogram was sprayed with diazotised sulphanilic acid and the coloured spot eluted and subsequently quantitated by spectrophotometry The accuracy of the method was indicated by a value of $\pm$ 9 8 %. KUTT *et al* (1964a) who have discussed the metabolism of phenytoin in several papers, give only a brief account of their method, from which it appears that it is related to Maynert s. With regard to the accuracy of their method, the investigators write "Reproducibility with standards was statisfactory deviations from mean were 0.3 for 5 µg spots, 0 7 for 10 µg spots and 0 8 for 15 µg spots"

PANALAKS (1963) describes a method for the determination of HPPH in urine, in which an individually conditioned blank value is determined in urine before starting the administration of phenytoin. This method cannot of course be used in patients under long term treatment.

In recent years, many studies on drug interaction have been published,

and in particular the influence of other drugs on the metabolism of pheny toin. (CUCINELL *et al* (1963 & 1965), KUTT *et al* (1964b & 1964c & 1964d), HANSEN *et al* (1967) and OLESEN (1966 & 1967a) In order to continue these studies, we have developed a method for determining HPPH in urine by means of thin layer chromatography

Method

3 ml of urine is mixed with 3 ml of concentrated HCl in a test-tube. The tube is closed with rubber test, through which capillary tube is inserted, and the test-tube then placed for 1 hour in a boiling water-bath. After cooling to room temperature, the contents are poured into a centrifuge tube and centrifuged for 2 minutes. 4.0 ml of the clear fluid in the upper layer is transferred by means of a pipett to a 15 ml centrifuge tube with a glass stopper and after adding 5.0 ml of chloroform-methyl acetate, 75 25 /v the tube is placed in a shaker for 5 minutes. The upper fluid layer is sucked off and 4 0 ml of the chloroform-methyl acetate layer evaporated to dryness n the water bath (60–70°), in stream of air The evaporation residue is washed to the bottom f the tube with 1 ml chloroform-methyl acetate and gain evaporated to dryness. The evaporation residue is dissolved in 100 µl chloroform-methanol 1 1 /v and 10 µl of this is applied to the chromatographic plate. Twodimensional thin-layer chromatography is performed in the same way as previously described (OLESEN 1967b & 1968).

Plates: 20 × 20 cm glass plates are covered with a 250 µm layer of Kieselgel G (Merck) to which Leuchtpigment ZS Super (Riedel de Haen) has been added. The plates are activated t 110 for 15 minutes.

Solvent Benzene, methanol, chloroform, concentrated ammonia water 11 22 66 2. This solvent is used for development in both directions. The chamber is lined with filter paper

Reference solution: 200 mg HPPH dissolved in 100 ml chloroform-methanol 1 1 v/v

Four samples or two duplicate samples, can be applied to plate, one in each corner 3µl of the reference solution are applied at the said points of each side (see fig. 1).

The HPPH spots from the samples are localized by means f the reference spots under U V lamp (254 nm), and the area covered by the spot is marked, as well as blank area of corresponding size, in which no bsorbent material is seen The HPPH spots and th blank spots are scraped off, and the powder poured into small centrif ge tubes, 1.5 ml of methanol are added, the tubes closed with a glass stopper and the powder extracted for about 2 minutes. After centrifugation, 1 ml of the supernatant is transferred by means f constriction pipette to semimicro quartz cuvettes (10 mm light path). 25 µl f 1 N-N OH are added to sample and blank, and the extinction measured at 243 nm before and after the ddition of 25 µl of 1 N-HCl to blank and sample.

Calculation of HPPH concentration in urine

$(E_{243nm\ alkaline} - E_{243\ acid}) \times 471$ mg/l HPPH

Results

Recovery and reproducibility

It was found that E_{43} alkaline $- E_{243\ acid}$ for a solution of pure HPPH in methanol obeyed Lambert Beer s law The conversion factor for cal-

Table 1

Recovery of HPPH added to different samples of urine. The means of duplicate determinations are given. The conversion factor 430 is the theoretical factor while 471 is the value found experimentally fo this factor

Added HPPH mg/l	ΔE_{243} = Mean	Found HPPH mg/l (ΔE_{243} = × 430)	Per cent Recovery
11 2	0.023	9 9	88 4
11 2	0.023	9.9	88 4
21 6	0.043	18.5	85 6
30.8	0.069	29 7	96.4
43 1	0.091	39 1	90.7
49.0	0.109	46.9	95.7
61 6	0.123	52.9	85.9
86.0	0.184	79 1	92.0
123 0	0.255	109 7	89.2
172.0	0.363	156.1	90.7
185.0	0.388	166 8	90.2
185.0	0.393	169.0	91 4
214.0	0.457	196.5	91.8
216 0	0.444	190.9	88.4
244 0	0.527	226.6	92.9
250.5	0.530	227.9	91.2
277.0	0.615	264.5	95.5
281 0	0.594	255 4	90.9
294.0	0.621	267 0	90 8
300.0	0.670	288 1	96.0
342.0	0.766	329 4	96.3
351 0	0.759	326.4	93.0

Mean recovery 91 4 % = 3.2 %.
Conversion factor for calculati g urinary co tent of HPPH = 471 s = 14

culating the concentration of HPPH expressed in mg/l (E × f = mg/l HPPH) was 38 7 determined on methanolic solutions with different known contents of HPPH Corrected for changes in volumes and the partition og HPPH between 6N HCl and chloroform-methyl acetate, (3 ml of urine are used $\frac{3}{4}$ of the hydrolyzed mixture is extracted, 84 4 % HPPH in the organic phase, $\frac{3}{4}$ evaporated $\frac{1}{10}$ on the plate, and finally the HPPH is extracted with 1.5 ml of methanol) the theoretical factor becomes 430, $(38.7 \times \frac{1}{3} \times \frac{4}{3} \times \frac{100}{84.4} \times \frac{4}{3} \times 10 \times 1.5)$.

The conversion factor used for calculating the urinary content of *HPPH* by means of $E_{243\,alkaline} - E_{243\,acid}$ was found by duplicate determinations on different urine samples to which HPPH (10–350 mg/l) was added

Table 2

Reproducibility of HPPH determinations in a sample of urine from a phenytoin-treated patient. 15 separate determinations are given.

	$AE_{243\ nm}$	mg/l HPPH (AE × 471)
1	0.605	285
2	0.606	285
3	0.581	274
4	0.585	276
5	0.590	278
6	0.595	280
7	0.584	275
8	0.586	276
9	0.603	284
10	0.603	284
11	0.582	274
12	0.598	282
13	0.637	300
14	0.586	276
15	0 630	297

Mean 281 7 mg/l $s = 7.9$
Coefficient of variation 2.8 %

(table 1) Mean recovery was 91 4 % ($s = 3$ 2 %) $n = 22$ duplicate determinations) the conversion factor was 471 ($s = 14$ $n = 22$)

The reproducibility was examined by performing 15 single determinations on a sample of urine from a phenytoin treated patient. The mean value was 281 7 mg/l HPPH, $s = 7.9$ coefficient of variation = 2.8 % (table 2).

Specificity

No absorbent spot was seen on the thin-layer plate in the area corresponding to HPPH when urine from patients or normal subjects who had not been treated with phenytoin was analyzed HPPH was distinguished from phenobarbital, phenytoin and OH-phenobarbital (fig. 1) A comparison between the U V extinction curves made with a pure sample of HPPH (a gift from Parke Davis & Co) and the U V extinction curves made with HPPH isolated from urine obtained from phenytoin-treated patients, showed good agreement (fig. 2) The substance stained with 4-aminophenazone-potassium ferricyanide (phenol).

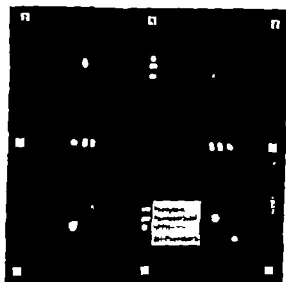


Fig. 1 Separation of HPPH from other agents in urine (phenytoin, phenobarbital and OH phenobarbital) by thin-layer chromatography A 20 × 20 cm Kieselgel covered glass plate is divided by means of channels before the chromatography The samples, $\frac{1}{10}$ of the concentrated extract, are applied to the corners of the plate. *1* and *3* Extract from hydrolyzed and non-hydrolyzed urine from patient treated with phenytoin. 2 and 4 Extract from non-hydrolyzed and hydrolyzed urine from a patient treated with phenobarbital. R Sites of application of the reference solution (OH-phenobarbital was not available). The spots were developed using mercuric sulphate reagent.

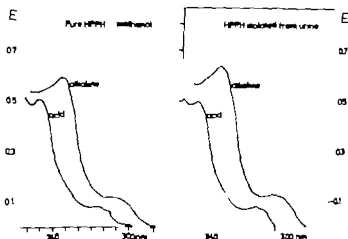


Fig. 2. The extinction curves in alkaline and acid methanolic solution of pure *HPPH* (14.5 mg/l) and HPPH isolated from urine obtained from a patient treated with phenytoin. The methanol was made alkaline by *adding 2.5 % 1 N NaOH and then* acidified by adding 2.5 % 3 N-HCl. (No correction has been made for the dilutions by the base and the acid. The curves were recorded by means of a Beckman DB spectrophotometer).

Discussion

When the fully-developed thin-layer plate was stained directly with phenol reagent, several spots showing different colours appeared, probably hormones (TOMPSETT 1964) None of the solvents tested by the investigator were able to separate HPPH satisfactorily from all the other stained components. However these coloured substances did not affect the U V determinations, so that this was taken as the method of choice.

In agreement with BUTLER (1957), we have found that HPPH is resistant to treatment with acid and heat, and that a prolonged heat treatment did not release further amounts of HPPH On the other hand with a constant period of heating, there was a clear reduction in the yield when the acid concentration in the mixture fell below 4 N

Even though the same solvent is used twice in the two-dimensional development of the plate, our experience is that a purer product is obtained in the two-dimensional development than in a one-dimensional development over a longer stretch.

Discussion

A two-dimensional thin-layer chromatographic method is described for determining 5-(p-hydroxyphenyl)-5-phenyl-hydantoin (HPPH) in urine.

3 ml urine was used for a single determination. The HPPH was released from glucuronic acid by acid hydrolysis, and after the substance was isolated it was determined quantitatively by U V spectrophotometry

Reproducibility Coefficient of variation was 2.8 / (15 single determinations on the same urine sample). Recovery was on an average, 91 4 / ($s = 3.2$ / $n = 22$ duplicate determinations on different urine samples to which HPPH was added in amounts varying from 10–350 µg/ml).

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Studies on Subcutaneous Absorption in Mice V
Absorption of Water Injected into the Skin of Normal and Oestradiol-treated Animals

By

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(Received November 9 1967)

The object of this study was to elucidate the significance of the connective tissue ground substance for the absorption of a volume of liquid injected subcutaneously

For this purpose the disappearance rates of subcutaneously injected volumes of various fluids, and the simultaneous clearance of ^3H_2O molecules contained in them have been examined in oestradiol-treated mice and compared with corresponding data for non-treated mice In the strain of mice used (Leo Stritt) treatment with oestradiol caused a considerable increase in the content of hexosamine (SCHMIDT 1958a) and uronic acid (unpublished) of the skin, while the permeability of the ground substance was reduced (COOPER & SCHMIDT 1957) and the hyaluronic acid polymerized (CHAIN & DUTHIE 1940 HVIDBERG & JENSEN 1959 SCHIFF & BURN 1961) whereas the fibrillary elements were unaffected (survey HVIDBERG SZPORNEY & LANGGÅRD 1963)

In addition the effect of hyaluronidase on the clearance of the volumes of fluid and the ^3H_2O molecules was examined in the oestradiol-treated mice as well as in the non-treated animals. Contrary to oestradiol-treatment addition of hyaluronidase caused a depolymerization of the hyaluronic acid (SCHMIDT 1958b) resulting in a reduction of the water binding capacity of the tissue (SCHOU 1959 HVIDBERG 1962 SECHER-HANSEN LANGGÅRD & SCHOU 1967a & b 1968)

Method

Albino male mice (Leo, Stritt) were pretreated with two subcutaneous injections of oestradiol monobenzoate 10 µg in 100 µl arachis oil, six and four days before the experiment. Under light halothane (fluothane ®) anaesthesia two symmetrical 5.5 cm^2 areas were

marked on the depilated skin of the back (cf SECHER HANSEN, LANGGÅRD & SCHOU 1967a). Eighty µl of distilled water 0.9 % NaCl or 10 % sucrose containing about 0.3 µc of ^3H_2O (New England Nuclear Corp.) per dose were injected subcutaneously within the right-side area. In other mice, the same solutions, but containing in addition 40 I.u. of hyaluronidase (penetrase Leo ®) per dose were injected In another series of experiments 80 µl of 0.9 % NaCl containing 0.5 µc of ^{131}I-human serum albumin was injected and in a corresponding group with 40 I.u. of hyaluronidase (penetrase Leo ®) per dose. For all of the groups mentioned parallel experiments were carried out in mice that had *not* been pretreated with oestradiol *Five* r fifteen minutes after the injections the animals were killed by decapitation. The two pieces of skin were excised and weighed, and the weight difference expressed in per cent of the 80 mg injected ("residual volume per cent"). The difference between the t tal contents of radioactivity of the skin pieces was determined (^3H_2O in a Packard Tri-Carb Liquid Scintillation Spectrometer model 3003 ^{131}I-albumin in a scintillation well type counter connected to an I.D.L Scaler 1700) and expressed in per cent of the dose injected ("residual radioactivity per cent").

Results

Fig. 1 shows that in oestradiol-treated animals the weight of the injected piece of skin *increases* during the first fifteen minutes after the injection *of distilled water whereas in the oestradiol treated mice addition* of hyaluronidase accelerates the volume clearance very significantly This effect of hyaluronidase is not observed in the untreated animals. A similar effect of hyaluronidase in the oestradiol-treated animals is seen after injection of 0.9 / NaCl (fig. 2) and 10 / sucrose (fig. 3)

Treatment with oestradiol significantly inhibits the clearance of the injected water molecules (fig. 4 5 & 6).

When hyaluronidase is added, a faster clearance of ^3H_2O molecules from pure water and 10 / sucrose (fig. 4 & 6) is seen, both in oestradiol-treated and in untreated animals. After the injection of 0.9 / NaCl, however hyaluronidase has no effect on the rate of disappearance of water molecules (fig. 5) In untreated animals the disappearance of ^3H_2O molecules takes place at a significantly higher rate in 0 9 / NaCl than in water and 10 / sucrose. After addition of hyaluronidase, however the disappearance rates in these solutions are the same (fig 4 5 & 6 c. SECHER HANSEN LANGGÅRD & SCHOU 1967b)

It appears from fig. 7a & b where data from the individual animal are shown that the 15 minute *residual volume per cent* for water and 0.9 / NaCl containing hyaluronidase, varies between −60 / and +90 / in the groups of oestradiol-treated mice, whereas *the residual radioactivity per cent* originating from ^3H_2O is very constant. For the same animals, fig. 8a & b show the relation between the percentage reduction of the injected volumes containing hyaluronidase, and the weight of the un-injected control sides (the thickness of the skin is taken as an indication of the

Fig. 1

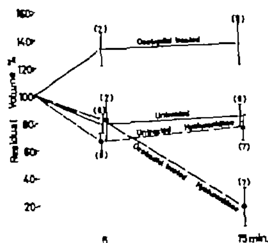


Fig. 1 Residual volume per cent 5 and 15 minutes after subcutaneous injections of 80 µl of distilled water with and without 40 I.u. of hyaluronidase per dose, to oestradiol pre-treated and normal mice. The standard error of the mean is indicated by vertical lines. Figures in brackets are number of experiments.

Fig 2

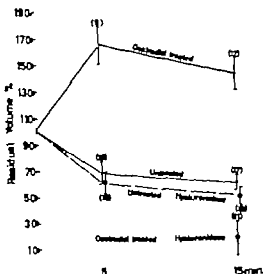


Fig. 2. Residual volume per cent 5 and 15 minutes after subcutaneous injections of 80 µl of 0.9 % NaCl with and without 40 I.u. of hyaluronidase per dose, to oestradiol-treated and normal mice.

Fig 3.

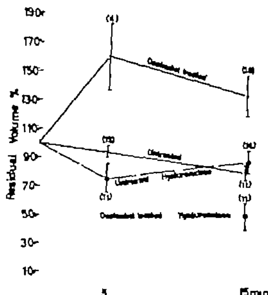


Fig. 3 Residual volume per cent 5 and 15 minutes after subcutaneous injections of 80 µl of 10% sucrose with and without 40 i.u. of hyaluronidase, to oestradiol-treated and normal mice.

Fig 4

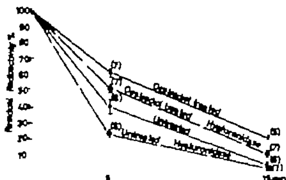


Fig. 4. Residual radioactivity per cent due to $^{3}H_2O$ 5 and 15 minutes after subcutaneous injections of 80 µl distilled water with and without 40 i.u. of hyaluronidase per dose, to oestradiol-treated and normal mice.

Fig 5

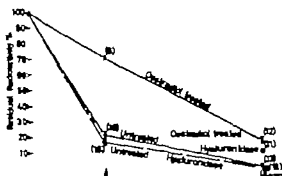


Fig. 5 Residual radioactivity per cent due to ^3H_2O 5 and 15 minutes after subcutaneous injections of 80 µl of 0.9 % NaCl with and without 40 u. of hyaluronidase per dose, to oestradiol-treated or normal mice.

effect of the oestradiol-treatment) The animals have been divided into five groups according to the weight of the control sides i.e. under 400 mg, 400–450 mg, 450–500 mg, 500–600 mg and over 600 mg. From this it appears that the thicker the skin the more rapidly the weight reduction of the injected piece of skin takes place, i.e. the more the animal has responded to the oestradiol treatment.

Fig 6

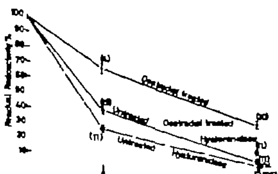


Fig. 6. Residual radioactivity per cent due to ^3H_2O 5 and 15 minutes after subcutaneous injections of 80 µl of 10 % sucrose with and without 40 I.u. of hyaluronidase per dose to oestradiol-treated and normal mice.

Fig 7a



Fig. 7a. Residual volume per cent ——— and residual radioactivity per cent ×——× 15 minutes after subcutaneous injections of 80 µl of distilled water containing 0.3 µc of ^3H_2O and 40 I.u. of hyaluronidase, to oestradiol-treated mice.

Fig 7b

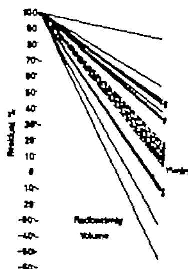


Fig. 7b. Residual volume per cent ——— and residual radioactivity per cent ×——× 15 minutes after subcutaneous injections of 80 µl of 0.9 % NaCl containing 0.3 µc of ^3H_2O and 40 I.u. of hyaluronidase, to oestradiol-treated mice.

Fig. 8a

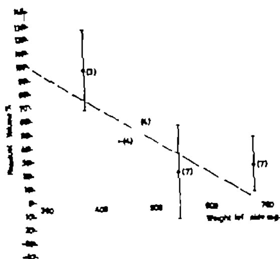


Fig. 8a. Residual volume per cent (ordinate) 15 minutes after subcutaneous injections of 80 µl of distilled water containing 40 I.u. of hyaluronidase, t oestradiol-treated mice in relation to the weight of the control side (abscissa).
The animals have been divided into 5 groups according to the weight of the uninjected control side, <400 mg, 400–450 mg, 450–500 mg, 500–600 mg and > 600 mg The standard *deviation* is given as vertical lines through the mean. F gures in brackets are number f experiments.

Fig 8b

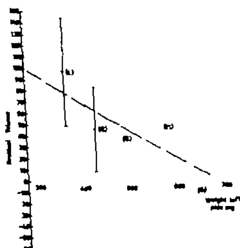


Fig. 8b. Residual volume per cent (ordinate) 15 minutes after subcutaneous injections of 80 µl of 0.9 NaCl containing 40 I.u. of hyaluronidase to oestradiol-treated mice in relation to the weight of the control side (abscissa).

Table 1

Residual volume per cent and residual radioactivity per cent, 15 minutes after subcutaneous injections of 80 µl 0.9 NaCl containing 0.5 µc of ^{131}I human serum albumin with and without 40 i.u. of hyaluronidase to oestradiol-treated and normal mice.

	0.9 % NaCl		0.9 % NaCl + Hyalyronidase	
	Residual Volume % mean ± s.e.m.	Residual Radioactivity % mean ± s.e.m.	Residual Volume % mean ± s.e.m.	Residual Radioactivity % mean ± s.e.m.
Normal	80 ± 7.8 (n = 9)	80 ± 3.7 (n = 9)	65 ± 9.8 (n = 8)	66 ± 2.5 (n = 8)
Oestradiol pre-treated	134 ± 9.8 (n = 6)	88 ± 1.3 (n = 6)	52 ± 13.2 (n = 4)	50 ± 6.1 (n = 4)

From table 1 it can be seen that in untreated animals the rate of disappearance of a 0.9 % NaCl volume and the rate of disappearance of ^{131}I human serum albumin molecules dissolved in it are the same. The same correlation is seen in the oestradiol treated mice when hyaluronidase is added to the injected fluid.

Discussion

When a volume of liquid is injected subcutaneously a rapid exchange of water molecules between the "depot" and the blood takes place, concomitantly with a much slower removal of the water surplus. With the same experimental set up as in the present study SECHER HANSEN, LANGGÅRD & SCHOU (1967a & b) demonstrated that *all* the water molecules injected were removed from the injection zone in the course of fifteen minutes, whereas no measurable decrease in the volume had taken place at this time. The exchange of water molecules is believed to be determined by the permeability of the tissue and by the local blood flow whereas the factors that determine the decrease in the volume are unknown. The significance in this respect of the permeability of the tissue and the local blood flow is not clear. The mechanically increased tension in the tissue presumably tends to diminish the surplus of liquid (cf. HVIDBERG 1960), whereas an aseptic inflammation caused by the injection trauma tends to increase the weight of the injected skin (BÁRÁNY 1932, FRANKE, BOATMAN, GEORGE & MOSES 1950, SECHER HANSEN, LANGGÅRD & SCHOU 1967c). Osmotic conditions may act in either direction (SECHER HANSEN, LANGGÅRD &

SCHOU 1967b) Because of the water binding capacity and the gel structure of the ground substance one or more ground substance factors may be of further importance as co-determinants of the clearance of a subcutaneously injected volume of fluid.

The results in fig. 1 2 & 3 which show identical conditions after injection of distilled water 0.9 % NaCl and 10 % sucrose, demonstrate the decisive significance of the ground substance for the decrease in volume. In oestradiol-treated mice the weight of the piece of skin injected *increases after* the injection by up to 60 % of the volume injected, presumably on account of an increased local water binding capacity brought about by the introduction in the tissue of the hypodermic needle and the volume per se, whereas addition of hyaluronidase implies that almost the whole of the volume injected in the oestradiol treated animals is removed in fifteen minutes In untreated mice the results are found to be "half way" between these outer limits, and addition of hyaluronidase to the injected volume has no effect. The results underline two features Firstly that the clearance of a subcutaneously injected volume may vary within very wide limits, depending on the state of the ground substance and secondly that the influence of the hyaluronidase on the decrease of volume is also decisively dependent on the state of the ground substance before the injection. This is further emphasized by the results in fig. 7a & b as well as in fig. 8a & b, showing a very considerable variation in the decrease of the volume in oestradiol treated animals after addition of hyaluronidase to the injected volume, so that the volume decreases at the quickest rate in mice who have responded the most to oestradiol. In a few of the animals there is a fall in weight of the piece of skin injected which exceeds the weight of the liquid injected, while at the same time 15 % of the radioactivity due to water molecules is still found at the injection site. The explanation must be that hyaluronidase in these animals has "mobilized" water from the surroundings of the injection site (fig. 7).

When the absorption rate of the radioactive water molecules in the solutions investigated (fig. 4 5 & 6) is slower in oestradiol-treated animals than in normal animals, this might be due either to the longer distance of diffusion or to a greater delay of diffusion in the oestradiol-treated mice. The fact that hyaluronidase increases the absorption rate very little indicates that the delay in the ground substance is chiefly due to the longer distance of diffusion.

Since the clearance of the injected volumes and the clearance of radioactive albumin molecules present therein are identical (table 1), and since albumin is known to be removed almost exclusively via the lymphatics (LEWIS 1921 FIELD & DRINKER 1931 HOLLANDER, REILLY & BURROWS 1961 LANGGÅRD 1964) it seems probable that a *volume* injected sub-

cutaneously is also cleared essentially through the lymph system. The slow decrease in the volume injected would thus be in agreement with the slow lymph drainage (SHREWSBURY 1939).

Summary

By studying oestradiol treated mice and the effect of addition of hyaluronidase to the injected solutions it is shown that the rate of the disappearance of a water surplus injected subcutaneously depends primarily on the state of the ground substance. Further that the influence of hyaluronidase on the disappearance of the volume also primarily depends on the state of the ground substance. The delay in the exchange of water molecules in the ground substance of the oestradiol-treated animals seems to be due to a longer diffusion distance rather than to a greater diffusion delay. The agreement found between the decrease in the volume and the disappearance of ^{131}I human serum albumin molecules contained therein suggests that subcutaneously injected volumes are cleared essentially through the lymphatic vessels.

Acknowledgements

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The Effect of Thyroxine and Adrenaline on *the Concentration of Hexose-Phosphates and High-Energy* Phosphate Compounds in Isolated Rat Diaphragm

By

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(Received October 19 1967)

In previous investigations, the potentiating effect of thyroxine-treatment on some of the metabolic effects of the catecholamines in the isolated rat diaphragm were studied. It was found that the lactate-forming effect of adrenaline was potentiated but not its glycogenolytic or phosphorylase activating effects (SVEDMYR 1965a & b 1966).

The mechanism and source of origin of this increased lactate formation however were therefore not localized In experiments on diaphragms from untreated rats, adrenaline reduced the glycogen content almost twice as much as it increased the lactate formation (SVEDMYR 1965a & b). Adrenaline considerably increased the concentration of hexose phosphates in muscle tissue, but also changed their relative distribution. This indicated that adrenaline also influenced the intermediary carbohydrate metabolism of skeletal muscle via pathways other than phosphorylase activation (BEVIZ, MOHME LUNDHOLM & SVEDMYR 1967) We therefore determined how treatment with thyroxine influenced the adrenaline effect on the hexose phosphate esters of the muscle. This was done in order to ascertain 1) if the origin of some of the increased lactate formation was to be found among these esters, and 2) if thyroxine treatment influenced the change of the relative distribution of the hexose phosphates produced by adrenaline.

Methods

Each animal of the thyroxine-treated groups was given a subcutaneous injection of 0.1 mg thyroxine for 7 days, which increased the oxygen consumption by about 25–30 % above the basal level, though the body weight was unaffected. They were then killed by blow on the neck. Three untreated and three thyroxine-treated rats were used for each set of experi

ments. The diaphragms were divided centrally one half serving as control and the other for incubation with adrenaline. The halves from the different animals were pooled and incubated in 15 ml Krebs-Henseleit bicarbonate buffer without any glucose, for 30 min. t 37 The incubation solution was bubbled with 95 / O_2 nd 5 CO_2 One group was incubated with adrenaline in a concentration of 10^{-6} g/ml. After 7.5 min. both groups muscle parts were frozen in Freon 11 containing carbon dioxide snow After weighing, the muscle was homogenized in 8 volumes ice-cold 6 % PCA. After centrifugation, the excess perchloric acid was neutralized with 5 M K_2CO_3. The precipitated potassium perchlorate was centrif ged in the cold. The extract was then nalysed for hexose phosphates and ATP ADP AMP CrP (creatine phosphate) as described previously (Bavtz *et al* 1965).

Results

It can be seen in table 1 that the ATP and CrP concentrations were significantly lower P < 0 05 in preparations from thyroxine treated rats. The ADP and AMP concentrations showed no definite change In the diaphragms from untreated animals the ATP and CrP concentrations increased after treatment with adrenaline (P < 0 05) while the ADP and AMP concentrations showed no definite change. The adrenaline effects in the thyroxine treated groups were somewhat lower than in the untreated groups, but the difference was not significant.

The results of the hexose phosphate analyses are shown in table 2. As seen in the table, there was no significant difference between the mean basal concentrations of G-1 P G-6-P F-6-P and F 1-6-P in preparations

Table 1

The effect of thyroxine and adrenaline on the concentrations f adenosinetriphosphate (ATP), adenosinediphosphate (ADP), adenosinemonophosphate (AMP) nd creatinephosphate (CrP) in isolated rat diaphragm. Concentration in µmol/g wet muscle. Mean ± s.m. (= 10). P = probability that the effect was due to chance.

	ATP	ADP	AMP	CrP
Untreated				
basal value	4 07 ± 0.27	1.08 ± 0 07	0 68 ± 0.03	5.26 ± 0.52
change fter adrenaline	+0.56 ± 0 25	+0.15 ± 0.14	+0.02 ± 0.04	+1 15 ± 0.46
	P < 0 05			P < 0.05
Thyroxine-treated				
basal value	3.27 ± 0.23	1 13 ± 0.06	0.65 ± 0 04	3.99 ± 0.27
change after adrenaline	+0.27 ± 0.31	+0.09 ± 0.11	+0.02 ± 0.05	+0 62 ± 0.52
Difference between basal value untreated thyroxine-treated	+0.80 ± 0 035	−0.05 ± 0 09	+0 03 ± 0.05	+1.27 ± 0.56
	P < 0.05			P < 0 05

Table 2.

The effect of adrenaline on the concentration of hexose-phosphates in isolated diaphragms from untreated and thyroxine-treated rats. G-1 P = glucose-1 phosphate G-6-P = glucose-6-phosphate F-6-P = fructose-6-phosphate F-1-6-P = fructose-1-6-phosphate. Concentration in μmol/g wet weight. Mean ± s.e.m. (n = 12).

	G 1 P	G-6-P	F-6-P	F-1-6-P
Untreated rats				
basal value	0.038 ± 0.003	0.238 ± 0.098	0.076 ± 0.008	0.380 ± 0.050
adrenaline	0.054 ± 0.005	0.486 ± 0.106	0.109 ± 0.007	0.684 ± 0.196
Increase per cent after adrenaline	48 ± 12	129 ± 25	46 ± 12	74 ± 22
Thyroxine-treated rats				
basal value	0.030 ± 0.003	0.237 ± 0.048	0.095 ± 0.010	0.365 ± 0.093
adrenaline	0.045 ± 0.004	0.464 ± 0.049	0.116 ± 0.008	0.658 ± 0.194
Increase per cent after adrenaline	61 ± 18	111 ± 21	29 ± 10	82 ± 29

from untreated and thyroxine treated animals. After treatment with adrenaline the hexose phosphate concentrations in the diaphragms of untreated and thyroxine-treated animals increased to the same extent. The dissimilarity in the percentage increase of G 1 P G-6-P F-6-P and F 1-6-P which was shown in the diaphragms of untreated animals was also observed in those of the thyroxine-treated animals

Discussion

After treatment with thyroxine the hexose phosphate concentrations in the rat diaphragm remained unchanged. Moreover thyroxine did not influence the increasing effect of adrenaline on the concentration of hexose phosphates or its effect on the relative distribution among the different hexose phosphates. These findings are in agreement with previous results (SVEDMYR 1965a & b) where it was found that treatment with thyroxine did not potentate the glycogenolytic or phosphorylase activating effects of adrenaline. The cause or origin of the increased lactate formation in thyroxine treated animals following adrenaline is thus obvious not dependent on changes in the hexose phosphate concentrations of the diaphragm.

CASTRO & MONACO (1953) found that adrenaline decreased the alanine and glutaminic acid content of the rat skeletal muscle proteins. The possibility that adrenaline stimulated lactate formation via some other

mechanism for example gluconeogenesis is therefore another alternative which should be considered

The concentrations of high-energy phosphate compounds (ATP and CrP) were diminished in diaphragms from thyroxine treated animals. The absolute ADP concentration was not significantly increased after thyroxine treatment, but the ratio $\frac{ATP}{ADP}$ was lower after this treatment (4 5 ± 0 40, and 3.2 ± 0 40 respectively difference = 1.3 ± 0 6 P < 0 05)

One important factor in the regulation of aerobic glycolysis is assumed to be the access to phosphate acceptors. The Pasteur effect, i.e. inhibition of glycolysis in the presence of oxygen, is assumed to depend partly on competition between aerobic and anaerobic processes for ADP and in organic phosphate (WILLIAMSON 1965). The increased aerobic glycolysis demonstrated after thyroxine treatment and which was also evident in previous experiments on the rat diaphragm (SVEDMYR 1965b) is assumed to be due to diminution of the ATP concentration in the cell, resulting from reduced ATP synthesis (uncoupling) and/or increased ATP utiliza tion (HOCH 1962). It is possible that the reduced levels of ATP shown in diaphragms of thyroxine treated rats, as compared with controls, plays an important role in the increased lactate formation both in the presence and absence of adrenaline.

Summary

Thyroxine given in a dose sufficient to increase the basal oxygen con sumption of the animals by 25–30 / did not affect the concentrations of G 1 P G-6-P F-6-P or F 1 P in the isolated rat diaphragm The ATP and CrP concentrations diminished after thyroxine treatment, but the con centrations of ADP and AMP were unchanged The effect of adrenaline (10^{-6} g/ml) on the concentration of hexose phosphates and on those of ATP and CrP was not altered by thyroxine treatment. The results are discussed with reference to previous experiments in which it was shown that in the rat diaphragm thyroxine potentiated the lactate forming effect of adrenaline but not its phosphorylase-activating effect.

Acknowledgement

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Effect of Ethanol on Rat Liver
VI A Possible Correlation between α-Glycerophosphate Oxidase Activity and Mitochondrial Size in Male and Female Rats fed Ethanol

By

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(Received November 8 1967)

A 15 / ethanol solution given to male rats over a period of several months has been shown to influence liver-mitochondrial oxidation (KIESSLING & TILANDER 1961 KIESSLING & PILSTRÖM 1966b) and the size and shape of the liver mitochondria (KIESSLING & PILSTRÖM 1966a KIESSLING & TOBÉ 1964) Thus the oxidation rates of pyruvate succinate, glutamate and sometimes β-hydroxybutyrate are reduced (KIESSLING & PILSTRÖM 1966b), whereas that of α-glycerophosphate is almost un changed (KIESSLING & PILSTRÖM 1966b) or even increased (KIESSLING & PILSTRÖM 1967 KIESSLING & PILSTRÖM, in press).

Morphologically the changes are of three types the first produces an elongated twisted mitochondrion with a narrow middle piece (KIESSLING & TOBÉ 1964), the second a greatly enlarged, rounded mitochondrion with disorganized and shortened cristae (KIESSLING & TOBÉ 1964) and the third, which is undoubtedly the most common one, is of the same appearance as the normal ones, only larger

In the present paper the hypothesis is brought forward that the last mentioned enlargement is a result of an adaptation in the liver to the new milieu created by ethanol and that the primary cause is an induction of the α glycerophosphate oxidase in the mitochondria.

Experiments and Methods

Male and female Wista rats from this laboratory' stock and male Sprague-Dawley rats (Anticimex, Stockholm) were used. When the animals ere 6 weeks old, they were into two groups, one of which received 15% (/v) ethanol solution and the

and a sucrose solution isocaloric with the ethanol taken by the ethanol-drinking rats. The animals were placed in separate cages and had free access to adequate solid food, the composition of which is given by KIESSLING & PILSTRÖM (1967).

At an age of 25 weeks the animals were practically full-grown. The weights of the alcohol-treated females were about 8 % lower than those of their water-drinking controls throughout the whole experimental period The corresponding figure for the male Wistar rats was 2 %. The alcoh l treated male Sprague Dawley rats also grew about 20 % more lowly than their controls, but this difference was no longer present during the 20th and 30th weeks.

The fluid consumption at the ge of 70 weeks was the same in the males whether they drank ethanol or water (6.5–7 ml/day/100 g body weight). The females drank 6.7 ml of ethanol solution per day The water consumption of the female controls was 7.8 ml.

After 200 days the animals were killed by decapitation and small pieces of peripheral parts of a liver lobe were cut out and put into ice-cold 20 % osmium tetroxide for 2 hours, then dehydrated in ethan l, embedded in epon, sections cut and examined in a Zeiss EM9 electron microscope, as described previously (KIESSLING & PILSTRÖM 1966a). The remainder of the liver was immediately chilled in ice-cold 0.25 *M* sucrose and liver mitochondria were prepared for manometric studies according to the method f ERNSTER & LÖW (1955).

Finally the mitcho dria were suspended in isotonic sucrose, so that 1 ml contained mitochondria from 300 mg of liver The respiratory rates with four substrates (pyruvate, succinate, α-glycerophosphate and β-hydroxybutyrate) were determined manometrically by the same procedures as described previously (KIESSLING & TILANDER 1961). The temperature was 30 and the gas phase was air Protein were determined according to CLELAND & SLATER (1953) by a colorimetric method depending on the biuret reaction.

Results

Fig. 1 shows that prolonged alcohol treatment of the rats brings about a marked increase in mitochondrial size in male Wistar and Sprague-Dawley rats but not in female Wistar rats. Female rats belonging to the Sprague Dawley strain were not studied

In table 1 the oxidation rates of liver mitochondria from male and female Wistar rats and from male Sprague Dawley rats treated with ethanol for 200 days are given. Here, the oxygen consumption has been calculated according to the protein content essentially the same result was obtained if the oxygen consumption was based on tissue weight. In male Wistar rats, prolonged ethanol treatment causes a significant decrease in the oxidation rates of pyruvate and succinate whereas that of α-glycerophosphate is significantly increased. In the females the corresponding oxidation rates are not influenced by the alcohol treatment. Alcohol treatment of male Sprague Dawley rats causes a slight increase in the oxidation rates of pyruvate and β-hydroxibutyrate an almost unchanged oxidation rate of succinate and a very marked stimulation of the rate at which α-glycerophosphate is oxidized

When pretreating normal rat liver mitochondria with hypotonic solu-

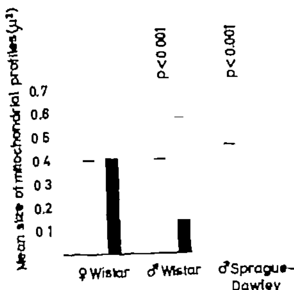


Fig. 1 The effect on mitochondrial size of giving ethanol to male and female rats for several weeks. The sex of the rats and the strain they belong to are given below the columns. ☐, controls drinking water and sucrose solution isocaloric with the ethanol consumed by the ethanol-fed rats. ■, rats drinking a 15% (/v) ethanol solution as the only drinking fluid. The treatment lasted for 200 days, at which time the liver samples were collected. Each column represents the mean value of at least eight animals.

tions, the respiration control was partly lost, the oxidation rate of NADH was markedly increased whereas that of α-glycerophosphate was unchanged as compared with untreated mitochondria. This excludes the possibility that an increased permeability to α-glycerophosphate is the cause of the increased oxidation rate in male alcohol-treated rats.

Discussion

Our previous observations that the oxidation rates of pyruvate and succinate were decreased in the mitochondria of alcohol-treated rats together with an enlargement of the mitochondria, led us to the assumption that this morphological change was to be considered as representing damage to the mitochondria. We think that this may still hold for the twisted or greatly enlarged types with disorganized cristae, which have been described in a previous paper (KIESSLING & TOBÉ 1964).

In addition to these two variations, we can also make out a third type with no structural disorganization these mitochondria only differ from

Table 1

The effect on mitochondrial oxidation rates of giving ethanol to male and female rats for several weeks.

Contr.: controls drinking water and a sucrose solution isocaloric with the ethanol consumed by the ethanol-fed rats.

Alc.: rats drinking a 15% (v/v) ethanol solution as the only drinking fluid. The treatment lasted for 200 days, at which time the mitochondria were prepared. The figures are mean values of 10–22 experiments ± S.E.

R.C. = respiratory control.

Animals		Oxygen consumption (µat. O_2/60 min./10 mg protein)				
		Pyruvate	R.C.	Succinate	β-hydroxy-butyrate	α-glycero-phosphate
Females (Wistar)	Contr.	4.2 ± 0.5	5.4	42.8 ± 0.6	18.4 ± 0.8	10.6 ± 0.3
	Alc.	23.8 ± 0.6	6.4	43.0 ± 0.6	18.6 ± 0.7	10.6 ± 0.3
	P	–		–	–	–
Males (Wistar)	Contr.	29.8 ± 0.6	5.9	39.2 ± 0.7	17.8 ± 0.7	7.9 ± 0.8
	Alc.	23.6 ± 0.9	5.2	30.8 ± 0.7	19.6 ± 0.8	10.4 ± 0.3
	P	0.01		0.01 – 0.001	0.2 – 0.1	0.01 – 0.001
Males (Sprague-Dawley)	Contr.	34.9 ± 0.9	6.2	53.6 ± 3.1	24.4 ± 0.8	8.7 ± 0.5
	Alc.	37.6 ± 1.1	6.8	51.8 ± 3.2	28.2 ± 1.0	14.7 ± 0.9
	P	0.1 – 0.05		0.7	0.02 – 0.01	0.001

normal mitochondria in their increased size. This type contributes most to the increase in mitochondrial size shown in fig. 1. Possibly this mitochondrion can later be transformed into any of the two pathological types described previously (KIESSLING & TOBÉ 1964), but in its present state it is greatly reminiscent, from a morphological point of view, of a normal mitochondrion except that it is larger.

Strangely enough this type of enlarged mitochondrion is common in male but not in female rats after prolonged alcohol treatment (fig. 1). This led us to examine the mitochondrial functions in the three experimental groups (table 1). From this table it is evident that the rate at which α-glycerophosphate can be oxidized changes after alcohol treatment of the animals, in a manner which parallels the changes in mitochondial size. Thus, the rate is significantly higher in the alcohol treated males of both strains, i.e. in the experimental groups with a marked increase in mitochondrial size, but is unchanged in the females, in which the size shows no increase after prolonged alcohol treatment. As the number of mito-

chondria is almost unaltered after alcohol treatment (KIESSLING & PILSTRÖM 1966b), the parallelism between the mitochondrial oxidation rate of α-glycerophosphate (table 1) and the increase in mitochondrial size (fig. 1) indicates that an induction of the α glycerophosphate oxidase in the mitochondria may explain the parallel increase in size.

This hypothesis prompts several questions. Why should prolonged ethanol treatment cause an increase in activity of this particular enzyme, why in males and not in females, and why do not the other enzyme activities studied increase in parallel? One very interesting observation about the effect of acute alcohol intoxication is the high rate of extramitochondrial conversion of NAD to NADH which is reflected primarily in an increase in the NADH/NAD ratio (SLATER & SAWYER 1964) and secondarily in high lactate/pyruvate and β-hydroxibutyrate/acetoacetate ratios (FORSANDER *et al* 1965). This is, of course, also true when ethanol is administered daily over a long period. A major system for NADH oxidation is the mitochondrial electron-transport system. As the membrane of the liver mitochondria is relatively impermeable to NADH (GREEN & CRANE 1958 LEHNINGER 1951) the re-oxidation of extra mitochondrial NADH by the electron-transport system has to be mediated by shuttle systems. The best known of these is that which involves α glycerophosphate-dihydroxyacetone phosphate (CHEFURKA 1954 ESTABROOK & SACKTOR 1958 BUCHER & KLINGENBERG 1958). A continuous increase in the NADH/NAD ratio above normal, caused by ethanol or by any other oxidizable compound present in unphysiologically high concentrations may of course, bring into play the full capacity of the cell to reduce this ratio to normal, including enzyme inductions of rate-limiting enzymes. It may thus be quite reasonable to expect an increase in the normally rather low activity of the mitochondrial α-glycerophosphate oxidase (table 1).

This increase amounts to a little more than 30 %, that is, up to the same level as found in control females. If this increase is sufficient, it may also explain why no increase in the activity of the α-glycerophosphate oxidase takes place when the females are treated with alcohol in the same way as the males.

Our working hypothesis, based on the results in fig. 1 and table 1 thus assumes that the mitochondrial α-glycerophosphate oxidase, being a rate limiting enzyme in the livers of male alcohol-treated rats, increases by means of enzyme induction, thus causing growth of the whole mitochondrion. Analogous to this mechanism is smooth endoplasm reticulum in liver which proliferates as a consequence of the phenobarbital-induced synthesis of drug metabolizing enzymes (REMMER & MERKER 1963 ORRENIUS *et al* 1965).

The hypothesis receives further experimental support from our previous observations of rats fed a diet rich in magnesium, choline and methionine simultaneously with the ethanol (KIESSLING & PILSTRÖM 1967) In these experiments ethanol alone causes a moderate increase in mitochondrial size, a marked decrease in the rate of pyruvate and succinate oxidation and an unchanged oxidation rate of α-glycerophosphate, as measured on a protein basis Addition of magnesium choline and methionine simultaneously with the ethanol, increases the mitochondrial size above that obtained with ethanol alone, as well as the oxidation rate of α glycerophosphate. The oxidation rates of pyruvate and succinate are, however still low

The simultaneous decrease in the oxidation rate of pyruvate and succinate in male Wistar rats, when calculated on a protein basis may actually even show a slight increase if they could be estimated as activity per mitochondrion In the male Sprague Dawley rats these activities may be increased significantly per mitochondrion

Two other shuttle systems have been discussed in the literature and may be considered in connection with the mitochondrial oxidation of extra mitochondrial NADH These are the β-hydroxybutyrate-acetoacetate couple (DEVLIN & BEDELL 1960 BOXER & DEVLIN 1961) and the malate-oxaloacetate couple (SACKTOR & DICK 1962 & 1964 BORST 1963 HASSINEN 1967) The existence of the first of these systems, however may be doubtful as the activity of β-hydroxybutyrate dehydrogenase is now known to be entirely restricted to the mitochondrial space. The meaning of the increased activity of this enzyme, which we have sometimes observed in our experiments (KIESSLING & PILSTRÖM 1967), is therefore obscure. The conclusion of HASSINEN (1967) that the malate-oxaloacetate couple may be of importance in the oxidation of extra mitochondrial NADH is based on the observation that addition of aspartate transaminase and malate dehydrogenase to mitochondria stimulates the respiration in the presence of extra-mitochondrial NADH This strongly suggests that the rate limiting enzymes in this shuttle system are located extra mitochondrially Our previous observation (KIESSLING & PILSTRÖM 1966b) that the malate dehydrogenase activity does not increase in liver after prolonged alcohol treatment of the rats, thus excludes the possibility that changes in this shuttle system contribute to the mitochondrial enlargement seen in fig. 1

Summary

The effects of prolonged alcohol treatment of male and female rats on the size and function of their liver mitochondria were studied

A significant increase of the mitochondrial size was found in male Wistar and Sprague Dawley rats but not in female Wistar rats. In addi tion, the mitochondria from the alcohol-treated female rats showed no changes in the oxidation rate of α-glycerophosphate, whereas this oxidation rate was significantly increased in the mitochondria from the alcohol-treated rats of both strains, as compared with the water-drinking controls.

In view of the results, a connection between induction of α-glycerophosphate oxidase in mitochondria and the increase in mitochondrial size has been suggested. A possible cause of the induction of this particular enzyme may be the extra mitochondrial increase in NADH when ethanol is oxidized. The initially relatively high activity of the α glycerophosphate oxidase in liver mitochondria from female rats, as compared with male rats, may explain why no corresponding increase in this enzyme activity and no increase in mitochondrial size take place in the females.

Acknowledgements

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I am indebted to Prof P E. Lindahl for placing the necessary facilities at our disposal, to Prof C. G Bernhard for giving us the opportunity of using the electron microscope and to Mrs. Ann-Sofie Berggren, Miss Maj-Britt Jansson and Miss Helga Möbius for skillful technical assistance.

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A Comparison of the Potency of Adrenaline and Noradrenaline in Delaying Absorption from Muscles

By

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(Received October 19 1967)

In view of the extensive use of local analgesia mainly obtained by the injection of local anaesthetics in combination with adrenaline or nor adrenaline, surprisingly little is known about the optimum concentrations of these vasoconstrictors.

As early as 1905 BRAUN recommended the addition of adrenaline in a concentration of 5 μg/ml. This concentration has been accepted even by authors of modern monographs on this subject (PITKIN 1953 BONICA 1954). The recommended concentration should be high enough to ensure a dependable decrease in absorption rate of the anaesthetic agent. It should not be higher than necessary since this might lead to systemic effects and also to a prolonged degree of local anaemia (and anoxia). This is not included in the recommendations given even in some recent textbooks of pharmacology For example GOODMAN and GILMAN (1965) mention two different concentrations of adrenaline in two chapters of the book (p 372 1 50,000–1 500 000 for adrenaline, 1 100 000 for noradrenaline p 511 1 20,000–1 100,000 for adrenaline). This uncertainty is reflected in the commercially available preparations of local anaesthetics in which the concentration of adrenaline and noradrenaline varies from 5 μg/ml (1 200,000) to 40 μg/ml (1 25 000).

Experiments are described below using the method of absorption studies described by SUND & SCHOU (1964) they were performed to answer the following questions 1) How high is the optimum concentration of adrenaline required to decrease absorption? 2) Is there a difference between the activity of adrenaline and noradrenaline? 3) To what extent does the addition of vasoconstrictors influence the physiological exchange processes?

Method

Male albino rats weighing 115–135 g were used. The absorption experiments with ^{14}C-sucrose and $^{3}H_2O$ were performed as described by SUND & SCHOU (1964).

Briefly 16 µl of the test solution (0.9 % NaCl in water with tracer doses of either ^{14}C-sucrose or $^{3}H_2O$) was injected into the exposed extensor quadriceps femoris muscle. At the end of the absorption period, the muscle was removed after ligation at each end, and the remaining radioactivity determined according to SUND & SCHOU (1964). In a Packard Tri-carb spectrometer. The remaining radioactivity was expressed as per cent of the remaining injected radioactivity as compared with standard countings.

The t-test was used for statistical calculation.

All concentrations of adrenaline and noradrenaline are expressed as the bases (the salts used were the bitartrates, quality NFN).

Results

Absorption of sucrose

In control experiments 37.5 ± 4.0 % (n = 10) of the injected sucrose remains at the injection site, 5 minutes after the injection. After 15 minutes, only 13.2 ± 1.9 % (n = 11) of the originally injected sucrose remains.

Adrenaline or noradrenaline added in the concentration of 1 µg/ml does not affect the absorption rate of sucrose (fig. 1). With 2.5 µg/ml an

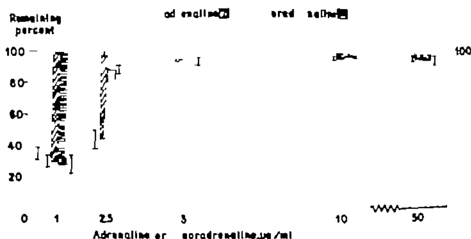


Fig. 1. The percentage of sucrose absorbed from rat extensor femoris muscles 5 minutes after injection of 16 µl 0.85 % NaCl solution with tracer amounts of ^{14}C-sucrose is indicated by the open bars (from below upwards), while the remaining percentage of sucrose is shown by filled or hatched columns from upper line and downwards (these values occur along the ordinate). The bars indicate experiments with 0, 1, 2.5, 5, 10 or 50 µg/ml adrenaline or noradrenaline in the injected solution (values shown on horizontal axis).

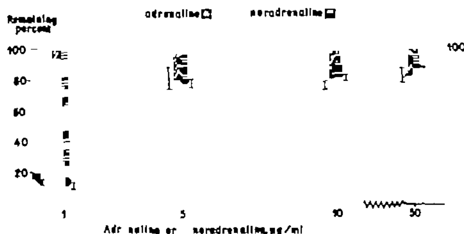


Fig. 2. The percentage of sucrose absorbed from rat extensor femoris muscles 15 minutes after injection of 16 µl 0.85 % NaCl solution with tracer amounts of ^{14}C-sucrose is indicated by the open bars (from below upwards) while the remaining percentage of sucrose is shown by filled or hatched columns from upper line and downwards (these values occur long the ordinate). The bars indicate experiments with 0, 1 5 10 or 50 µg/ml adrenaline or noradrenaline I the injected solution (values shown on horizontal axis).

effect of noradrenaline is apparant as 88 4 ± 3.3 / (n = 4) of the sucrose remains after 5 minutes, while adrenaline in the same concentration is without any effect (44 1 ± 7 2 / left, n = 4).

With both adrenaline and noradrenaline in concentrations of 5 10 or 50 µg/ml, a significant depression of sucrose absorption is found as only about 5 / of the injected sucrose is removed from the injection sites during the first 5 minutes.

The results of 15 minutes absorption experiments confirm the above findings (fig. 2). Approximately 20 / of the injected sucrose is cleared with the addition of 5 10 or 50 µg/ml adrenaline or noradrenaline as compared with 87 / in control experiments.

Absorption of ^3H_2O

The net clearance rate for injected water (^3H_2O) is faster than for sucrose. However in experimental determinations of the remaining per centage of ^3H_2O 9 minutes after the injection and with or without 1 5 10 or 50 µg/ml adrenaline or noradrenaline (fig. 3 and table 1) a pattern similar to that observed in the sucrose experiments was observed. 1 µg/ml of adrenaline or noradrenaline barely affected the clearance rate while a significant inhibition of the absorption was found with all the three higher

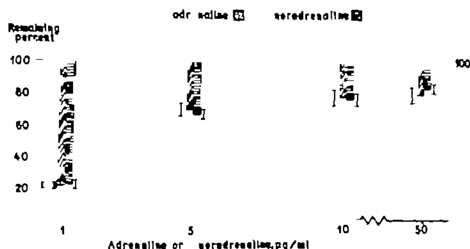


Fig. 3 The percentage of ^3H_2O absorbed from rat extensor femoris muscles 9 minutes after injection of 16μl 0.85% NaCl solution with tracer amounts of ^3H_2O is indicated by the open bars (from below upwards), while the remaining percentage of sucrose is shown by filled or hatched columns from upper line and downwards (these values occur along the ordinata). The bars indicate experiments with 0, 1, 5, 10 or 50 μg/ml adrenaline or noradrenaline in the injected solution (values shown on horizontal axis).

concentration levels of the vasoconstrictors. As is evident from the figures in table 1 the vasoconstrictor effect increased – or exerted a more prolonged effect – with increasing concentrations of the vasoconstrictors.

Discussion

The experiments reported above show that a significant inhibition of sucrose absorption from rat muscles is obtained with 5 μg/ml of either adrenaline or noradrenaline. Very little, if any further effect is obtained even by increasing the concentrations of the vasoconstrictors by tenfold to 50 μg/ml. There was no significant difference of effect between adrenaline and noradrenaline in equivalent concentrations.

The experiment with ^3H_2O as a test substance was performed to get an impression of the extent to which the vasoconstrictors influence the physiological exchange processes. From these experiments it is evident that the higher the concentrations of the vasoconstrictors the greater the inhibition of the exchange processes is likely to occur.

In conclusion these experiments point to the 52 year old statement of BRAUN (1905) Adrenaline should be used in a concentration of 5 μg/ml with injection solutions for local anaesthesia. There is no reason for in-

Table 1

Rate coefficients for all the absorption experiments, calculated on the assumption that the absorption process can be described as a monoexponential functio (first order reaction). The coefficient is calculated as $k = \frac{\log y - \log y_t}{0.4341 \times t}$ where y_o and y are the percentage remaining mounts at the start and at the end of period of the absorption process, while t indicates the duration of the period (in m utes) The figures in the table are these coefficients multiplied by 10^3 ($k \times 10^3$)

Adrenaline µg/ml	Tritiated water		Sucrose-14C	
	0–3 minutes	3–9 minutes	0–5 minutes	5–15 minutes
0	24.2	13.0	19.7	10.8
0.5	25.1	23.7	14.3	8.1
1.0	24.4	13.1	21.0	14.0
2.5	–		16.4	–
5.0	3.1	4.4	1.0	1.6
10.0	3.5	2.6	0.8	2.2
50.0	–	2.6[1])	1.0	1.4
Noradrenaline µg/ml				
0.5	29.2	10.1	–	12.5[2])
1.0	26.6	15.9	19.6	12.7
2.5	–	–	2.5	–
5.0	4.2	4.6	2.8	1.8
10.0	4.7	2.2	0.6	1.5
50.0	–	2.1[1])	1.2	0.8

1) Calculated for the period 0–9 minutes.
2) Calculated for the period 0–15 minutes.

creasing this concentration further as the absorption is hardly further affected. On the contrary higher concentrations seem undesirable due to their unfortunate depression of the physiological exchange processes between the tissue cells and the blood.

Summary

The effect of adrenaline and noradrenaline was compared in absorption experiments from rat muscles using either ^{14}C-sucrose or $^{3}H_2O$ as test substances. The optimal concentration for both vasoconstrictors w

found to be 5 μg/ml. With this concentration a satisfactory absorption depression was obtained while the physiological exchange processes were least affected

Acknowledgements

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Distribution and Excretion of Various Mercury Compounds after Single Injections in Poultry

By

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(Received March 30, 1967)

Large amounts of mercury in wild birds in Sweden have been reported by several investigators BORG (1958) ULFVARSON (1965), BORG *et al* (1965) WESTERMARK (1965), BERG *et al* (1966) and LIHNELL & STENMARK (1967). In order to interpret these reports it is important to know something about the toxicology of different mercury compounds in birds.

The excretion and distribution of organic mercury compounds used as fungicides has been studied mainly in mammals, e.g. by SWENSSON (1959a & b) ULFVARSON (1962) and BERLIN *et al.* (1963). Data on birds on the other hand are scanty

SMART & LLOYD (1963) fed hens with seed treated with *methyl mercury dicyandiamide* and analysed some organs when the experiment was terminated. The rate of excretion was not measured BORG (1958) and BORG *et al* (1965) fed pheasants and some other birds with seed treated with *methyl mercury dicyandiamide* until the birds died They analysed the Hg content of the liver kidneys and muscles in order to establish the lethal concentrations in these organs TEJNING (1965) in limited experiments fed hens and cocks with seed treated with *methyl mercury dicyandiamide* and studied the mercury content in eggs and in the organs. HELMINEN *et al* (1966) fed pheasants with seed treated with *methoxyethylmercury silicate* They reported the distribution of mercury in several organs. ULFVARSON (1965) fed pheasants with *methyl mercury dicyandiamide* and also made a rough calculation on the rate of excretion. A constant excretion rate of slightly less than 3 percent of the simultaneous mercury content per day was found.

In the present investigation the excretion rate of three organic mercury

found to be 5 μg/ml. With this concentration a satisfactory absorption depression was obtained, while the physiological exchange processes were least affected

Acknowledgements

This investigation was supported by the Foundation for the advancement of Medical Science, Copenhagen, and Miss P A. Brandts Foundation Mrs. Vivi Halling gave perfect technical assistance

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Table 1

Distribution of mercury between different organs in white leghorn cocks after single injections of some mercury compounds.

Substance		Days after injection	Blood ng/g	Blood $\frac{s}{\sqrt{N}}$	Liver ng/g	Liver $\frac{s}{\sqrt{N}}$	Kidneys ng/g	Kidneys $\frac{s}{\sqrt{N}}$	Muscle ng/g	Muscle $\frac{s}{\sqrt{N}}$	Brain ng/g	Brain $\frac{s}{\sqrt{N}}$	Injected amounts μg/kg	Number of animals
$Hg(NO_3)_2$	Exp. I	6	5	1	390	140	402	166	8	4	23	6	500	2
		15	4	4	286	164	2680	190	3	0	7	3		
	Exp. II	10	4.9	0.6	1416	273	1973	590	18.2	4.8	33.1	3.0	500	4
		20	1.4	0.1	906	178	1004	123[1])	4.6	0.8	9.8	0.9		
Me Hg OH	Exp. I	6	7390		9280		9930	–	1320		1 10	–	7000	1
		15	2900	230	3880	148	5240	700	1850	1260	3010	495		2
	Exp. II	10	4680	340	7220	390	8100	280	4270	450	3760	110	6000	4
		20	1940	100	3510	198	3960	377	2070	85	1780	94		
Fenyl HgOH	Exp. II	10	55.7	15.5	19670	4630	13340	5690	443	395	265	56	4000	3
		20	11.6	1.8	2813	1543	3976	408	25.4	3.8	85	15		4
M O Et Hg OH	Exp. I	6	22	1	900	122	2250	280	58	18	701	10	4000	2
		15	6	1	521	459	1450	304	34	13	95	33		
	Exp. II	10	6	4	2900	870	4330	1174	150	26	194	13	6000	4
		20	4	3	862	162	1507	439	25	7	36	8		

1) 3 djur

compounds and one inorganic mercury compound was determined in white leghorn cocks The mercury distribution in several organs was also studied.

Materials and Methods

The mercury compounds were synthesised from $Hg^{203}O$ and the mercury analysis of the organs made by γ-counting with the scintillation counting technique as described previously by SWENSSON *et al* (1959a) and ULFVARSON (1962).

The substances investigated were mercury (II) nitrate, phenyl mercury hydroxide, methyl mercury hydroxide and methoxyethyl mercury hydroxide.

Young white leghorn cocks weighing about 1 kg were used. Th cocks were injected i travenously with aqueous solutions of mercury with a single dose corresponding to about one fifth of the LD50. After injection of the mercury compound the cocks were kept in single cages on a wire net. The feces was collected on a plastic covered plate.

In one preliminary experiment the cocks had free access to food and water This resulted in severe contamination of the feces from the food which made the mercury analysis of doubtful value. Therefore only the concentrations in the organs were determined. In the second experiment the animals were taught to eat their 24 hours supply in 2 hours. During the remaining part of the 24 hours period, the animals had free access to drinking water By this procedure contamination of the excrements with food could be avoided. Faeces were collected once every day immediately before feeding and placed in polyethylene tubes and analysed with no further treatment for radioactivity The excretion of mercury per 4 hours was determined.

I the preliminary experiment 4 animals were used for each substance. Phenyl mercury hydroxide was excluded in this experiment. 2 animals from each group were killed after 6 days and the remaining animals fter 15 days.

In the second experiment 8 animals were used for each substance. 4 animals were killed after 10 days and the remaining 4 animals fter 20 days.

The animals were dissected and the following organs taken blood, liver kidney breast muscle and brain. Each organ was placed separately in a polyethylene tube nd analysed for radioactivity without further treatment.

Results

The mean of the concentrations of the organs in the different groups are given in table 1 as ng mercury per g of organ together with the standard error of the means. When the animals died before the end of the experiment, they were not analysed. The number of animals analysed on each occasion is shown in table 1 The data on excretion are given in tables 2 to 5 In these tables the excretion is given in μg of mercury per kg of body weight for each day during the observation period as the means for 8 animals during the first 10 days and separately as the means of the excretion for the 4 animals which were studied during the whole observation period. The concentration of mercury remaining in the bodies were calculated by subtracting the amount excreted from the amount given.

Table 3

Excretion of mercury in white leghorn cocks after a single injection of methylmercury hydroxide, 6000 μg/kg body weight

Days after inj.	Excreted Hg/day ΔX (μg/kg)		Remaining Hg (X) (μg/kg) (4 animals)	Accumulated excretion % of injected amount	$\frac{\Delta X}{X}$ (4 animals)	$\frac{\Delta X}{X^2}$ (4 animals)
	8 animals	4 animals				
1	182	182	6000	3.0	$3.1\ 10^{-2}$	$5.2\ 10^{-6}$
2	129	157	5818	5.1	2.7	4.6
3	81.6	81.5	5661	6 6	1 4	2.5
4	113	107	5579	8.4	1 9	3 4
5	74.4	75.5	5472	9 6	1 4	2.6
6	101	120	5396	11.3	2.2	4.1
7	126	110	5276	13 4	1	4.0
8	94.6	108	5166	14 9	2.1	4 1
9	122	103	5058	16 9	2.1	4.2
10	151	146	4955	19 4	3.0	6.0
11	–	124	4809	22.0	2.6	5 4
12		98.2	4685	23 7	2.1	4.5
13	–	61.9	4587	24.7	1 4	3.0
14		66.1	4525	[illegible]5.8	1.5	3 3
15	–	50 0	4459	26.7	1 1	2.5
16	–	79.6	4409	28 0	1.8	4 1
17		47.5	4329	28.8	1 1	[illegible].5
18		56.8	4281	29.8	1.3	3.0
19	–	42.4	4224	30.5	1 0	2.4
20		39.8	4182	31 1	1.0	2.4
						$3\ 7\ 10^{-6}$

Table 4

Excretion of mercury in white leghorn cocks after single injection of phenylmercury hydroxide, 4000 μg/kg body weight

Days after inj.	Excreted Hg/day ΔX (μg/kg)		Remaining Hg (X) (μg/kg)	Accumulated excretion of injected amount	$\frac{\Delta X}{X}$	$\frac{\Delta X}{X^2}$
	8 animals	4 animals	(4 animals)		(4 animals)	(4 animals)
1	1160	1200	4000	29.6	3.0 10^{-1}	7.5 10^{-3}
2	601	354	2800	44.9	2.0	7.1
3	312	337	2246	52.8	1.5	6.7
4	263	343	1909	59.5	1.9	10.0
5	210	220	1566	64.9	1.5	9.5
6	189	154	1346	69.7	1.[illegible]	8.9
7	108	83.8	1192	72.4	0.74	6.2
8	112	105	1108	75.3	1.0	9.0
9	98	97.5	1003	77.8	1.0	10.0
10	108	115	905	80.5	1.4	15.5
11	–	67.5	790	83.3	0.93	11.8
12	–	55.5	722	84.7	0.80	11.1
13	–	49.6	666	85.9	0.77	11.7
14	–	41.9	616	87.0	0.70	11.3
15	–	37.6	574	88.0	0.68	11.8
16	–	32.5	536	88.8	0.63	11.7
17	–	32.5	503	89.6	0.67	13.3
18	–	35.1	470	90.5	0.77	16.3
19	–	30.8	435	91.3	0.74	17.0
20	–	24.0	404	91.9	0.62	15.3
						11.1 10^{-3}

Table 5

Methoxy ethyl mercury hydroxide, 6000 μg/kg body weight

Days after inj.	Excreted Hg/day ΔX (μg/kg) 8 animals	4 animals	Remaining Hg (X) (μg/kg) (4 animals)	Accumulated excretion % of Injected amount	$\frac{\Delta X}{X}$ (4 animals)	$\frac{\Delta X}{X^2}$ (4 animals)
1	120	1540	6000	26.1	$2.6 \cdot 10^{-1}$	$4.4 \cdot 10^{-5}$
2	1170	1380	4460	49.5	3.2	7.2
3	690	684	3080	61.1	2.3	7.5
4	409	390	2396	67.7	1.7	7.1
5	327	341	2006	73.8	1.9	9.5
6	189	177	1645	76.8	1.1	6.7
7	220	201	1468	80.2	1.5	10.2
8	147	164	1267	83.0	1.4	11.0
9	93.3	107	1103	84.8	1.1	10.0
10	109	99.2	996	86.4	1.1	10.1
11	–	58	897	87.4	0.73	8.1
12	–	70.2	839	88.6	0.95	11.3
13	–	32.5	769	89.2	0.48	6.2
14	–	26.6	736	89.6	0.42	5.7
15		26.6	709	90.1	0.43	6.1
16	–	21.4	682	90.4	0.37	5.4
17		13.7	661	90.7	0.24	3.6
18	–	14	647	91.0	0.39	6.0
19		8.6	626	91.2	0.16	2.6
20		8.6	617	91.3	0.16	2.6
						$7.1 \cdot 10^{-5}$

This has been made only for the 4 animals which were allowed to go through the whole experimental period of 20 days. The accumulated excretion during the whole period has also been calculated Finally the daily excretion as μg of mercury per kg of body weight has been divided by the simultaneous mercury concentration and also by the square of this concentration. These figures are discussed below

Discussion

Distribution

In table 6 the ratios between the concentrations in some organs have been calculated The distribution between the different organs is seen to be fairly constant at different times after the injection with some remarkable exceptions. Although the concentration of mercury in the kidneys of the animals which received phenylmercury hydroxide decreases with time the concentration in relation to other organs increases. This has been demonstrated in rats ULFVARSON (1962) and BERLIN *et al.* (1963). A similar finding is seen with mercury (II) nitrate though in this case the conclusion is very uncertain, since in the second experiment the opposite trend is found.

The distribution between blood and brain is rather similar for mercury (II) nitrate, phenyl mercury hydroxide and methoxyethyl mercury hydroxide, the brain concentration being between 5 and 10 times higher than the blood concentration. Methyl mercury hydroxide brings about the highest absolute brain concentration of mercury but the ratio between blood and brain is lowest being only about 1 or less In experiment 1 where the animals were analysed after 6 and 15 days there is an indication of a delayed increase in the brain-concentration of methyl mercury hydroxide, the ratio brain blood concentration increases and it can be seen in table 1 that the brain concentration increases despite the decrease in blood concentration. This is consistent with the findings in rats by BERLIN *et al* (1963) and SWENSSON & ULFVARSON (1968)

As has also been shown in rats SWENSSON *et al* (1959a & b) ULFVARSON (1962) BERLIN (1963) the distribution in the cocks of methyl mercury hydroxide is comparatively even in all organs. With all the other compounds investigated the concentrations in the blood and muscle are very low and the liver and kidney concentrations fairly high to very high This is also illustrated in fig. 1 where the concentration of mercury in the different organs, divided by the kidney concentration is represented by blocks in a logarithmic diagram.

Table 6

Ratios between concentration in different organs.

Compound	Exp.	Days after inj.	*Muscle liver*	*Blood liver*	*Brain: blood*	*Liver: kidney*
$Hg(NO_3)_2$	Exp. I	6	0.021	0.013	4.6	0.97
		15	0.010	0.014	2	0.11
	Exp. II	10	0.013	0.0035	6.7	0.72
		20	0.0055	0.0015	7.0	0.90
M Hg OH	Exp. I	6	0.14	0.80	0.16	0.93
		15	0.48	0.75	1.0	0.74
	Exp. II	10	0.59	0.65	0.80	0.89
		20	0.59	0.55	0.92	0.89
F Hg OH	Exp. II	10	0.023	0.0028	4.7	1.5
		20	0.0069	0.0041	7.3	0.71
M O Et Hg OH	Exp. I	6	0.098	0.024	9.1	0.40
		15	0.065	0.012	15.8	0.36
	Exp. II	10	0.060	0.010	7.5	0.58
		20	0.029	0.046	9.0	0.57

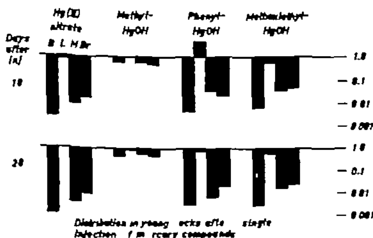


Fig. 1 The concentration of mercury the different organs (B = blood, L = liver M = muscle, Br = brain), divided by the kidney concentration, is represented as blocks in logarithmic diagram.

Excretion

During the first few days the excretion of methoxyethyl mercury hydroxide was the highest of all the compounds investigated. Hence the accumulated excretion is always highest as compared with the other compounds. Thus after 10 days in this case only about 10 % of the injected amount of mercury remained in the bodies. The animals that received phenyl mercury hydroxide retained about 20 % in their bodies after 10 days and in those who got mercury (II) nitrate about 40 % was left, whereas about 80 % of methyl-mercury hydroxide still remained in the bodies at the same time.

The organic mercury compounds were given in doses of the same order of magnitude, about 4 to 6 mg per kg of body weight. Mercury (II) nitrate on the other hand was given in a dose which was about 10 times lower. Since the excretion in relation to the remaining mercury concentration changes considerably during the observation period, and in all cases decreases, a comparison between the excreted amounts at a definite period after the injection which does not take into account the amount injected may be inadequate.

As was shown by ULFVARSON (1962) the excretion data obtained from rats injected with methylmercury hydroxide give a rather good fit to an exponential curve of the general type

$$x = x_0 \cdot e^{-kt}$$

Where x = the concentration of mercury in the body at any time
x_0 = the concentration immediately after the injection
t = the time after the injection
k = a proportional constant which may be approximated by the expression $\Delta x/x$, where Δx means the excretion per unit time, e.g. during one day and x means the simultaneous mercury concentration. The dimension of k is time^{-1}.

It was also shown in rats that phenylmercury hydroxide, methoxiethyl mercury hydroxide and mercury (II) nitrate were not excreted according to this simple mechanism ULFVARSON (1962).

In tables 2 to 5 it can be seen that $\Delta x/x$ varies systematically for all mercury compounds investigated, since apart from smaller occasional variations it decreases all the time.

The decrease is comparatively small for methyl mercury hydroxide but rather substantial for the other mercury compounds. $\Delta x/x^2$ on the other hand, shows much less variation for all compounds. This indicates a hyperbolic excretion mechanism of the general form

$$\frac{1}{x} = k \quad t + \frac{1}{x_0}$$

where x, x_0 and t have the same meaning as above

k = proportional constant which may be approximated by the expression $\Delta x/x^2$ The dimension of k is conc.$^{-1}$ time^{-1}

In figure 2 the concentration of the mercury retained each day during the observation period is plotted against time after injection. In the same diagram the hyperbolas of the above form are also shown with k equal to the mean of $\Delta x/x^2$ for all days. A fairly good fit to the experimental data is found for all compounds. The data obtained with methylmercury

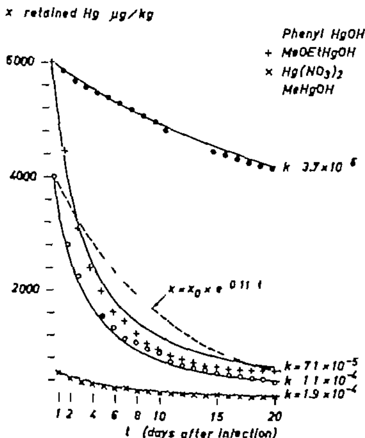


Fig. 2. Retained mercury concentration in the whole bodies of white leghorn cocks t different times after single injections of mercury compounds.

hydroxide on the other hand give almost as good a fit to an exponential curve. In this range of concentration and constant of excretion it is difficult to decide whether an hyperbola or an exponential curve gives the best fit to the experimental data. $\Delta x/x^2$ shows however slightly less variations than $\Delta x/x$ which indicates that even with methylmercury hydroxide the hyperbola is to be preferred.

In the figure an exponential curve with k equal to the mean of $\Delta x/x$ for phenylmercury hydroxide is also shown in order to demonstrate the poor agreement with the corresponding experimental data in this case.

The question as to whether a hyperbolic excretion mechanism for the compounds investigated implies a bimolecular reaction prior to the elimination of the mercury containing molecules or is the result of a combination of several unknown processes has to be left open.

Since this type of excretion mechanism serves the purpose of describing the data obtained better than the exponential mechanism, it has been used here.

Conclusions

The distribution between the organs of the mercury compounds investigated has been found to be principally the same as in rats. The number of observations is small and therefore transfer between the organs may not show up in these experiments. It has however been noticed that phenylmercury hydroxide is eliminated more slowly from the kidneys than from other organs.

The excretion data fit a hyperbolic curve much better than an exponential curve The means of the rate constants during the observation period are for mercury (II) nitrate 1.9 10^{-4} for phenylmercury hydroxide 1 1 10^{-4} for methoxyethyl mercury hydroxide 7 1 10^{-5} and for methyl mercury hydroxide 3 7 10^{-6} day^{-1} (μg mercury per kg of body weight)$^{-1}$ Thus mercury (II) nitrate has the highest excretion rate according to these data. There is however not much difference between mercury (II) nitrate, phenylmercury hydroxide and methoxyethyl mercury hydroxide, whereas methyl mercury hydroxide is excreted at a much slower rate.

To get an idea of what these rates signify the biological half-lives may be calculated on different assumptions. With $k = 10^{-4}$ day^{-1} (μg/kg)$^{-1}$ and $x_0 = 10000$ μg/kg (10 ppm) half of the mercury content in the body will be excreted in 1 day With $k = 4$ 10^{-6} and x_0 the same as above, the corresponding time will be 25 days. If $x_0 = 1000$ μg/kg (1 ppm) the times of excretion of half of the mercury content in the body will be 10 and 250 days respectively i e. with excretion constants mentioned above.

Summary

The excretion and distribution after a single injection of mercury (II) nitrate, methylmercury hydroxide, phenylmercury hydroxide and methoxyethyl mercury hydroxide, tagged with Hg^{203} has been investigated in white leghorn cocks by means of the scintillation technique. The amount of mercury given to the animals varied according to the acute toxicity of the different mercury compounds, and was calculated to correspond to about 1/5 of LD50 of each compound. In the main experiment, 8 cocks were used for each substance. The excretion was followed each day after the injection until the 20th day On the 10th day half of the animals were killed and the organs taken for analysis of radioactivity On the 20th day the remaining animals were killed and analyzed in the same way

The ratio between the concentrations in the different organs was found to be fairly constant at both times after the injection. However phenyl mercury hydroxide was eliminated less rapidly from the kidneys than from other organs. For all investigated mercury compounds except methylmercury hydroxide it was found that the concentration of mercury is highest in the liver and the kidneys and much lower in other parts of the body Methylmercury hydroxide, on the other hand, is rather evenly distributed in the body This is in agreement with what has previously been found in rats.

Methylmercury hydroxide is excreted in an amount per unit time which is fairly proportional to the simultaneous mercury concentration in the body This implies a logarithmic excretion mechanism and is consistent with what has been found previously in rats The other three mercury compounds are not excreted according to this mechanism but are better described if hyperbolas are fitted to the data.

The data from methylmercury injected animals also agrees with such a mechanism and it is also indicated that the agreement is still better with this than with the exponential mechanism.

Mercury (II) nitrate phenylmercury hydroxide and methoxyethyl mercury hydroxide are excreted at about the same rates, with a rate constant of about 10^{-4} (µg/kg) $^{-1}$ day^{-1} while methyl mercury hydroxide is excreted at a much slower rate, the rate constant being about 4 10^{-6} (µg/kg) $^{-1}$ day^{-1}

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Distribution and Excretion of Mercury Compounds in Rats over a Long Period after a Single Injection

By

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Rats were given single injections of organic mercuric compounds, SWENSSON *et al* (1959a) Long term exposure to such compounds was studied by allowing the animals to have free access to drinking water containing the labelled substances, SWENSSON *et al* (1959b)

It was found that the excretion of methyl mercury hydroxide was much less than that of phenyl mercuric hydroxide and mercury (II) nitrate during the first few hours after the injection of the respective compounds SWENSSON *et al* (1959a) It was also demonstrated that the organic compounds gave much higher mercury concentrations in the blood and in the organs studied, than the inorganic compounds, SWENSSON *et al* (1959b). A distribution equilibrium was indicated between the blood and the brain, SWENSSON *et al.* (1959b).

Subsequently a simple excretion mechanism was assumed by ULFVARSON (1962) for the alkyl mercuric salts, according to which the excretion per unit time is proportional to the simultanous concentration of mercury in the body The excretion data of phenyl mercuric hydroxide and mercury (II) nitrate did not agree with this simple theory since it appeared that these compounds were changed in the body of the experimental animals during the observation period ULFVARSON (1962). These conclusions were drawn from experiments with long term exposure. The mercury compounds were administered in small doses every other day during the experimental period i.e. 18 days.

BERLIN & ULLBERG (1963) followed the retention and accumulation of mercury compounds in animals by the use of an autoradiographic technique. In these studies the animals were followed for 16 days.

Longer observation periods should be of great value in establishing the excretion rate and eventual changes in the distribution between the organs. Due to the rather short half life of the radioactive mercury isotope Hg^{203} it has been difficult to carry out such studies with the usual isotope technique. In this study, however, we used mercury compounds labelled with Hg^{203} and with a high specific activity.

Materials and Methods

The mercury compounds were synthesized from $Hg^{203}O$ as described previously SWENSSON *et al.* (1959 a). (The specific activity of the oxide was about 200 mCi/g). The mercury analyses of the organs were made by γ-counting with the scintillation technique as described previously SWENSSON *et al.* (1959 b).

The substances investigated were mercury (II) nitrate, phenyl mercuric hydroxide and methyl mercuric hydroxide.

Female albino rats weighing about 200 g were used. The animals while under narcosis were injected intravenously with 100 µg of mercury per animal in 0.5 ml of water. The rats were killed after 1, 2, 4, 9, 16, 40, 83, and 169 days. Three rats were used for each observation day.

After killing the rats the following organs were taken: blood, liver, kidney, testis, brain, skin and the remainder of the body. The brain was dissected into five parts: lobus olfactorius, cerebellum, pons + medulla oblongata, cerebral cortex and the residue of the brain. Each organ from the three rats of each group and each part of the brain was placed separately in a polyethylene tube and analysed for radioactivity without any further treatment.

During the whole period analyses of the radioactivity in the whole body of the living animals were made in a large well scintillation crystal.

Results

The analyses are given in table 1, 2 and 3 as ng mercury/g of organ. Although the number of animals at each time is only three the standard error is calculated for each mean in order to get an idea of the dispersion. The animals which received mercuric (II) nitrate all died after the 83rd day and therefore no concentration values could be obtained after that time.

The concentration values are also plotted against time in logarithmic diagrams in fig. 1, 2 and 3. To avoid difficulties in interpretation only the concentration in part of the brain has been presented in the figures, i.e. cerebellum.

The result of the radioactivity measurements of whole bodies are reported in table 4 as percent of the initial dose present at different times after the injection.

Table I

Mercuric nitrate. Concentrations in g/g of organ. Mea f three animals.
The figures below the means indicate the *andard error*

Days after injection	Brain: Lobus olf	Brain: Cerebellum	Brain: Pons + med. obl.	Brain: Cerebral cort.	Brain: Residue of the brain	Blood	Liver	Kidney	Testis	Skin	Residue of the body
1	56	37 4	37 4	38 8	38 9	146 42	1665 75	13500 1900	57 15	126 32	111 40
2	63 15	48 8	55 12	48 10	44 11	69	976 140	10400 300	63 6	91 13	116 11
4	51 8	39 6	39 7	29 3	29 7	41 3	382 9	11700 1900	55 5	76 9	55 4
9	55 23	23 9	29 6	20 4	22 7	28 7	141 27	14700 2000	43 4	57 6	33 1
16	36 1	20 3	23 3	13 3	15 2	27 2	67	8100 1100	27 1	37 4	24 3
40	6 1	3 1	3 1	2 1	2 1	6 5	12 2	1610 160	6 2	7 1	6 3
83	4 4	0.7 0.2	0.8 0	0.38 0.04	0.51 0.02	0.9 0.3	2 1	305 54	0.9 0.3	3 1	– –

Table 2

Phenylmercuric hydroxide Concentrations in ng/g of organ. Mean three animals. The figures below the means indicate the *standard error*

Days after injection	Brain: Lobus olf	Brain: Cere-bellum	Brain: Pons + med. obl.	Brain: Cerebral cortex	Brain: Rest	Blood	Liver	Kidney	Testis	Skin	Rest
1	62	38	32	31	27	1154	2177	20900	59	104	309
	14	3	3	4	1	60	240	1000	2	5	64
2	33	24	23	19	17	168	598	16600	37	65	98
	3	1	1	1	1	4	56	100	1	2	9
4	27	4	25	19	18	77	397	22000	45	72	61
	2	1	1	1	1	5	27	300	5	10	5
9	26	21	23	14	15	35	161	25400	31	42	32
	4	1	1	2	1	9	21	1600	2	6	6
16	33	15	20	9	12	47	83	16300	23	40	24
	3	1	4	1	1	9	8	100	3	3	2
40	10	7	7	6	6	26	18	2270	6	25	12
	3	1	1	2	1	5	0	10	1	1	2
83	0.4	0.8	0.3	0.7	0.4	5	~1	315	0.75	4.2	–
	0.5	0.2	0.2	0.3	0.1	3	0.5	60	0.13	2.0	
169		–	–	–	–	3.3	1.1	41.2	0.30	3.2	2.5
						0.6	0.1	0.9	0.05	0.5	1.0

Table 3

Methylmercuric hydroxide. Concentrations in µg/g of organ. Mean from three animals.
The figures below the means indicate the *standard error*

Days after injection	Brain: Lobus if	Brain: Cerebellum	Brain: Pons + med bl.	Brain: Cerebral cortex	Brain: Rest	Blood	Liver	Kidney	Testis	Skin	Rest
1	182	161	127	135	117	4116	1127	2925	175	296	338
	12	13	4	4	7	220	52	260		85	–
	153	181	145	166	133	3034	735	2479	168	318	356
	48	7	2	1	9	120	3	370	3	1.5	32
4	220	218	146	186	171	2673	781	3280	206	1221	351
	10	7	5	1	9	120	31	260	20	820	20
9	173	217	143	212	182	2131	585	2927	153	216	332
	10	10	1	9	10	130	1	190	12	108	18
16	170	176	148	170	179	1795	480	2616	135	374	291
	16	20	22	31	13	190	70	186	13	270	46
40	78	65	36	62	68	437	159	1629	35	535	117
	4	0	2	3	3	53	8	70	2	250	46
83	4	5	3	5	5	32	12	412	2.6	48	–
	2	1	1	1	2	16	3	170	0.6	36	
169		–				8.1	1.8	63	0.7	162	70
						0.8	0.3	7	0.1	74	5

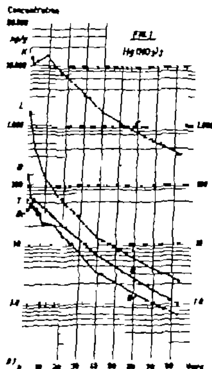


Fig. 1 The concentration of mercury (II) nitrate in different organs (Br = brain, B = blood, L = liver, K = kidney, T = testis) at various times.

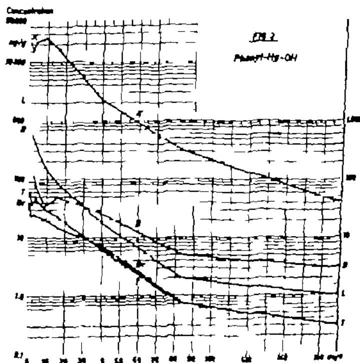


Fig. — The concentration of phenyl mercuric hydroxide in different organs (Br = brain, B = blood, L = liver, K = kidney, T = testis) at various times.

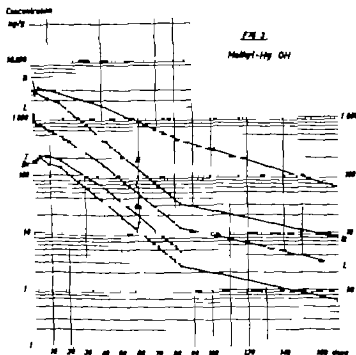


Fig. 3. The concentration of methyl mercuric hydroxide in different organs (Br — brain, B — blood, L — liver K — kidney T — testis) at various times.

Table 4

Whole body analyses (means of all animals) and estimated "biological half-lif of the compounds at different times after the injection f the mercury compounds.

Days after injection	Mercury (II) nitrate		Phenyl mercuric hydrox.		Methyl mercuric hydrox.	
	% of initial dose retained	biological halflif (days)	of initial dose retained	biological halflif (days)	% of initial dose retained	biological halflif (days)
9	29	5	30	5	67	16
16	17	6	22	7	57	20
40	6.8	10	5 1	11	34	26
83	2.7	16	3.5	17	20	36
169		–	3.5	(35)	10	51

) C lculated from the amount of mercury retained.

Discussion

Excretion rate Only if the excretion per unit time is proportional to the simultanous mercury concentration in the body and the proportional constant remains unchanged during the observation period is it correct to use the idea biological half life to characterize the excretion rate of the substances It has already been established that the excretion mechanism is not as simple as that at least for phenyl mercuric compounds or mercury (II) compounds (ULFVARSON 1962). However since the half life is easy to understand, it will be used here on the assumption that it will not be looked on as a constant, but only as a measure of the excretion rate during a given period of time.

In table 4 it can be seen that in all cases the excretion is most rapid immediately after the injection The excretion rate is about the same for mercury (II) nitrate and phenyl mercury hydroxide varying with a "biological half life from about 5 days when the observation period is 9 days, to about 10 days when the observation is extended to 40 days. For methyl mercuric hydroxide the "biological half-life« is about 16 days judging from what is retained after 9 days and 26 days when the retention after 40 days is considered. This is in perfect agreement with previous reports, when a different method of measurement was used (ULFVARSON 1962).

From table 4 it is also clear that when only a slight amount of the injected mercury is left in the body the excretion is very slow This is also shown in figures 2 and 3

Recently it has been shown in poultry by SWENSSON & ULFVARSON (1968) that a good description of the excretion of mercury compounds after a single injection is obtained if it is assumed that the excretion at each moment is proportional to the square of the simultaneous concentration in the whole body The concentration in the whole body decreases hyperbolically

Thus $1/x = k \cdot t + 1/x_0$, where x = the concentration of mercury in the whole body while x_0 = the initial concentration of mercury t = time and k = a proportional constant. If $1/x$ is plotted in a diagram against t, a straight line is obtained if x decreases hyperbolically In fig. 4 the concentration of mercury in the whole body is plotted against time, using an inverted scale ($1/x$) on the ordinate. As can be seen in the figure, the excretion for all three mercury compounds, rather closely follows the theoretical straight lines. Phenyl mercuric hydroxide and mercury (II) nitrate apparently have very similar rate constants of about 10^{-3} $(\mu g/kg)^{-1}$ day^{-1} while methyl mercuric hydroxide has a lower rate constant, about 10^{-4} $(\mu g/kg)^{-1}$ day^{-1} These rate constants are all higher than the corresponding constants found in poultry SWENSSON & ULFVARSON (1968).

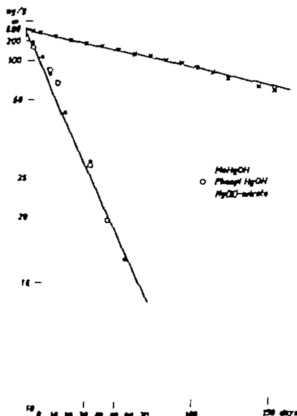


Fig. 4 Remaining mercury concentration in the whole body of rats after single injection of 500 g/g (Scale 1/x).

Distribution As can be seen in tables 1 2 and 3 and in the figures, the relations between the concentrations of mercury in the different organs is not constant, but varies for all compounds investigated.

The blood concentration changes rapidly from high values in the animals killed 1 day after the injection to much lower values in those animals killed subsequently At the same time the kidney concentration goes up and reaches a maximum on the 9th day for mercury (II) nitrate and phenyl mercuric hydroxide and on the 4th day for methyl mercuric hydroxide

The initial blood concentration (1 day after the injection) is highest for the two organic mercurials, phenyl and methyl mercuric hydroxide. The blood concentration remains comparatively high for methyl mercuric hydroxide while phenyl mercuric hydroxide returns to the same level as that of mercury (II) nitrate This may indicate a rapid decomposition

of phenyl mercuric hydroxide or rather the phenyl mercury ion. Such a decomposition has also been indicated previously (ULFVARSON 1962).

After a certain time the *relations* between the concentrations in the different organs seem to become stabilized and remain rather constant through the remainder of the period. The time it takes for the compounds to arrive at such an equilibrium varies. After about 10 days, methyl mercury hydroxide and mercury (II) nitrate seem to reach this state, while changes in the *relations* seem to continue for between 30 and 40 days with phenyl mercuric hydroxide.

After about 100 days the concentrations of the mercury compounds seem to reach levels which are of the same order of magnitude as the background levels normally found in control animals. It is however interesting to note that the *relation* between the concentrations in the different organs still remains the same. This seems to imply that there is still a dynamic equilibrium between the molecules containing mercury in the different organs, (or if this is not the case, that the rate of elimination from each organ at this stage is the same.)

The concentration of mercury compounds in the brain and testis follows a somewhat different picture than that of other organs. For methyl mercuric hydroxide and mercury (II) nitrate the concentration of mercury reaches a maximum after some time, while the concentration in other organs with the exception of the kidneys always decreases.

With methyl mercuric hydroxide the maximum concentration is reached on the 4th day and with mercury (II) nitrate on the 2nd day. The elimination of mercury from the brain and the testes then becomes slower than in the other organs with all mercurials investigated, up to between the 10th and 20th day. These findings are in agreement with those of BERLIN & ULLBERG (1963) using a autoradiographic technique.

The concentration in different parts of the brain is rather similar for all mercury compounds, though a somewhat higher concentration in *lobus olfactorius* is found during the whole observation period.

It should be kept in mind that the method used only allows of the measurement of the mean concentration in an organ or part of an organ investigated. Highly localized concentration differences can not be found with this technique.

Summary

Rats were given single subcutaneous injections of methyl mercuric hydroxide, mercury (II) nitrate and phenyl mercuric hydroxide. The elimination of the compounds and the concentration in the organs at

different times were followed by means of isotope techniques, for almost 6 months.

The rate of excretion changes during the observation period. A calcul ated "biological half life" therefore will be different from time to time. If the elimination during the first 9 days is considered the "half life" is 5 days for mercury (II) nitrate and phenyl mercuric hydroxide and 16 days for methyl mercuric hydroxide. After this the excretion rate becomes slower and slower

The distribution in the organs varies during the first part of the period. The blood concentration decreases rapidly for all compounds, while the concentrations in the kidneys increases and reaches a maximum after some days. The same holds true for the brain and testis when mercury (II) nitrate and methyl mercuric hydroxide are considered The elimination from the kidneys, the brain and the testes is slower than from other organs for all compounds.

The concentrations in different parts of the brain are rather similar though a somewhat higher concentration in lobus olfactorius is indicated

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Acta pharmacol et toxicol 1968 26 284–292

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Study on Combined Treatment with Phenobarbital and Diphenylhydantoin

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(Received October 13 1967)

In recent years, knowledge has accumulated, showing that treatment with certain drugs can inhibit or accelerate the metabolic inactivation of other drugs (NETTER 1962 REMMER 1962). After it had become evident that these experimental findings may also have some clinical significance, some uncertainty arose whenever combined treatment with two or more drugs had to be given.

The problem seemed of particular importance in the treatment of epilepsy where combinations of antiepileptic drugs as well as combinations of antiepileptic and other drugs are common In the first category combinations of phenobarbital and diphenylhydantoin are widely used *Phenobarbital is known to be a strong* inducer of microsomal enzymes and is thus capable of accelerating the metabolic breakdown of many other drugs. In 1963 CUCINELL *et al* first showed in dogs with very high doses that treatment with phenobarbital was able to decrease the half life of diphenylhydantoin to about ½ of the initial values. Two years later the same investigators (CUCINELL *et al* 1965) pointed out that the plasma concentrations of diphenylhydantoin were lower in patients simultaneously treated with phenobarbital than in other patients receiving only the hydantoin. This finding, however could not be reproduced generally by other investigators (LIVINGSTON 1966 MOLHOLM HANSEN *et al* 1967)

It seemed therefore of interest to investigate the mutual interactions of both drugs under rather "pure" experimental conditions in a species resembling man with regard to rates of metabolic inactivation and also using doses corresponding to those given in the patient.

Methods

All experiments were performed in dogs, mostly mongrels, of both sexes, aged from 1 to 5 years, and weighing between 18 and 32 kg. These were bought from animal dealers shortly before the experiments. The dogs were only used once. They were fed a standardized dog food ("Doggy", Lavens kemiske fabrik) and once a week they received meat and bones.

Four series of experiments were performed:

1. 3 dogs (male, 1 year, 20 kg; male, 1½ years, 31 kg; male, 1 year, 27 kg). The half-life for phenobarbital was determined after intravenous injection of 10 mg/kg (brought into solution with sodium hydroxide). 3 weeks later the dogs received oral doses of 3 mg/kg (tablets) once daily at 4 p.m. during 30 days. During this period, serum phenobarbital concentrations were determined twice weekly at 8 a.m. The half-life was again determined after the last dose (without a further injection) as well as 1 and 3 weeks after the end of the treatment and then at longer intervals (usually once a month) until the value obtained before treatment was reached. In the latter cases, a dose of 10 mg/kg was always injected intravenously.

2. 3 dogs (male, 1 year, 19 kg; male, 4 years, 32 kg; male, 1½ years, 28 kg). Corresponding experiments were performed with diphenylhydantoin. The half-lives were determined after intravenous injection of 10 mg/kg (brought into solution with sodium hydroxide). Treatment consisted of daily doses of 10 mg/kg orally (tablets) at 8 a.m. for 30 days. Twice weekly serum concentrations were determined 2 and 4 hours after the administration.

3. 3 dogs (male, 2 years, 18 kg; female, 2 years, 23 kg; male, 5 years, 28 kg). In this series, dogs were treated with daily doses of 3 mg/kg phenobarbital orally for 30 days. The serum concentration was determined twice weekly at 8 a.m. Before, during, and after treatment, the half-life of diphenylhydantoin was determined at weekly intervals.

4. 4 dogs (male, 1 year, 20 kg; female, 1 year, 29 kg; female, 5 years, 28 kg; female, 2 years, 22 kg). The half-lives of both phenobarbital and diphenylhydantoin were determined. Three weeks later the dogs received 10 mg/kg diphenylhydantoin at 8 a.m. and 3 mg/kg phenobarbital at 4 p.m. orally for 30 days. During treatment, the serum concentrations of both drugs were followed as described under 1 and 2. After the end of the treatment, the half-lives of both drugs were again repeatedly determined (immediately after cessation of treatment, 1 and 3 weeks later and then once monthly until the initial values were reached).

For the determination of the half-life of phenobarbital blood samples were taken 1, 2, 4, 6, 24, 48, 72 and 96 hours after the intravenous injection of the drug. In the case of diphenylhydantoin, the determination of the concentrations at 30 min., 1, 2, 4 and 6 hours after the injection was sufficient. The dogs were always injected at 8 a.m. The concentrations determined by the method described below were plotted on semi-logarithmic paper against time and the half-life was estimated graphically.

The half-lives were usually determined only once, before the start of the treatment in order to avoid any influence of repeated doses on the subsequent metabolism of the drugs. It should be mentioned however that RÜMMERT & BÜCKERT (1962) did not find an enzyme-inducing effect with intravenous doses of phenobarbital in dogs. With regard to the constancy of half-lives values in the individual animal, we have some previous experiments in which the half-lives for the two drugs were determined twice, in addition to those of some dogs from the present study in which the half-lives were determined twice after normalization to the initial value. In 8 dogs, the difference between 2 determinations of the half-life of diphenylhydantoin taken at intervals of between 2 weeks and 2 months averaged +0.11 ± 0.4 hours. In three other dogs, half-lives of phenobarbital were determined twice again at

intervals of 2–8 weeks. Here the half-lives had fallen, respectively from 90 to 80, from 35 to 33 and from 53 to 50 hours. Thus, a certain enzyme-inducing ffect cannot be excluded in the case of phenobarbital, though, on the other hand, the half-lives of both drugs seem to be rather constant for a single individual.

Phenobarbital and diphenylhydantoin were extracted from serum d separated by the method of WESTERINK & GLERUM (1964). The principle of this method is extraction by chloroform, the two drugs being then separated on a buffered Celite 545-column by elution with chloroform. The first 2 ml of the eluate are discarded. Diphenylhyd ntoin appears in the next 10 ml from which it is reextracted into 0.01 N NaOH and then determined by UV-spectrophotometry by measuring the extinction difference between 235 and 260 nm. Phenobarbital appears in the following 30 ml of chloroform, is reextracted into 0.45 N NaOH and then determined by measuring the extinction difference between 255 and 275 nm. To ensure comparability of results, this procedure was also adopted when only one of the two drugs had to be determined.

Results

Phenobarbital series Two of the three dogs in this series showed a marked fall in the half life immediately after cessation of treatment, or one week later (fig. 1) The minimal values were determined one and three weeks after treatment 25 and 29 hours as compared to 52 and 63 hours before treatment. It took more than 3 and 7 months, respectively for these dogs to reach the initial half life. While these dogs demonstrate the well known capacity of phenobarbital to accelerate its own metabolic breakdown the third dog showed an opposite effect Here, the half-life had fallen from 74 hours before to 57 hours just after treatment but it had already reached the initial value one week later and was even prolonged 3 weeks and two months after treatment.

Diphenylhydantoin-series The three dogs in this series had initial half

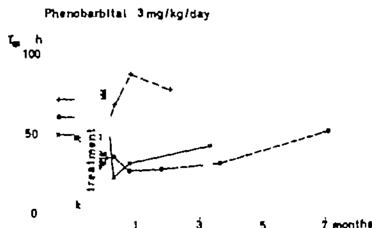


Fig. 1 Half-life of phenobarbital in dogs before and after treatment with a daily dose of 3 mg/kg orally for 30 d ys.

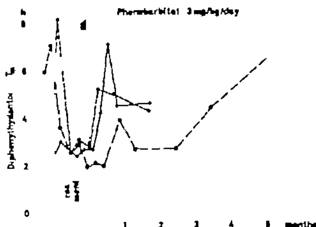


Fig. 2. Influence of treatment with 3 mg/kg/d y phenobarbital on the half-life of diphenylhydanto n in dogs.

lives between 2.8 and 3 7 hours and showed a slight trend towards a prolongation immediately after treatment. This was followed by a downward variation one week later and after three weeks, the half lives had already returned to between 2.8 and 3 8 hours.

Influence of phenobarbital-treatment on the half-life of diphenylhydantoin (fig. 2) The results with the three dogs in this group were quite different. In the dog with the lowest initial value, the half-life did not change during treatment but rose from 2.5 to over 7 hours in the following three weeks. It then remained at a level considerably above the initial value. In the second animal the half-life of diphenylhydantoin already rose from 3 4 to more than 8 hours in the first week of treatment, then fell back to values of about 3 hours during the remainder of the phenobarbital-treatment, after which it rose again to values of about 5 hours in the two months after treatment In the third dog, with a rather long half-life of 6 to 7 hours initially this fell to about 2 hours during the treatment, and it was not until 5 months later that the initial value was reached again.

Combined treatment with phenobarbital and diphenylhydantoin (fig. 3) In this group the half-lives of diphenylhydantoin appeared to be some what lowered after cessation of treatment, but recovered to the initial level within two months. The only exception was a dog with a conspicuously short half-life of 1.3 hours at the start here the half-life had risen to 3 hours after treatment and remained at this level

The half-lives of phenobarbital were shortened considerably in two of the four dogs at the end of treatment, but reached or exceeded the initial values as early as one to three weeks later In the third dog the half

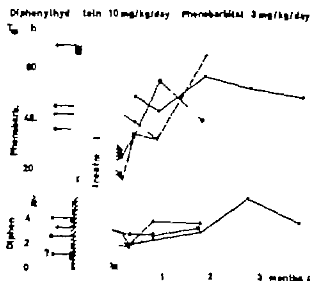


Fig. 3 Half-lives of phenobarbital and diphenylhydantoin in dogs before and after treatment with both phenobarbital (3 mg/kg/day) and diphenylhydantoin (10 mg/kg/day) for 30 days.

life fell from 70 to 43 hours during treatment and fell further within the next three weeks, but had almost recovered after two months. The last dog showed higher values after than before treatment

Serum concentrations during treatment In the 6 dogs treated with phenobarbital only the serum concentration rose within the first 4–5 days

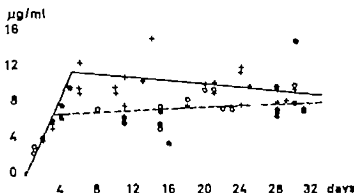


Fig. 4. Course of the serum concentration f phenobarbital during treatment with phenobarbital alone (+ ——) or with both phenobarbital and diphenylhyda toin (O,) in dogs. The daily dose of phenobarbital was 3 mg/kg, that of diphenylhydantoin 10 mg/kg, both were given orally The curves represent determinations in 6 nd 5 animals, respectively

after which a plateau of 10–12 μg/ml was reached. This, however levelled off slowly during further treatment (fig. 4) again indicating the capacity of phenobarbital to accelerate its own metabolism.

In five dogs treated with the combination of phenobarbital and diphenylhydantoin, the course of the phenobarbital serum concentration was somewhat different. This already levelled off when an average concentration of 7 μg/ml was reached after 3 days, but had a tendency to rise so that after about 30 days of treatment the concentrations in both groups were about equal

The diphenylhydantoin concentrations determined 2 and 4 hours after the oral administration always remained below 4 μg/ml regardless of whether phenobarbital was given.

Discussion

The results of the present study corroborate on the one hand the well-known fact that phenobarbital is capable by induction of microsomal enzymes in the liver to accelerate its own metabolic inactivation, as well as that of other drugs, particularly diphenylhydantoin. On the other hand it is apparent that this phenomenon cannot be regarded as a rule without exceptions. In a considerable number of the experiments we found no alterations or even longer half lives after treatment than before, and these were even maintained for some time. Further the cases behaving "typically" i e., reacting with a shortening of half-lives showed a rather large variation with regard to the time necessary for normalization to the initial value this varied from a few weeks to more than half a year

The initial situation seems to have some bearing on the divergent results. It were particularly the animals with high half lives at the start that reacted with a marked acceleration of metabolic breakdown, and, vice versa, dogs with short half lives at the start often showed a tendency to a prolongation in spite of the treatment.

The explanation may lie in the animal material used by us. This dogs of unknown origin coming into our hands shortly before the experiments, may have had some contact with foreign compounds which may have had some positive or negative effect on the drug-metabolizing enzymes. An example is the dog in fig. 3 with an initial half-life of only 1.3 hours for diphenylhydantoin It is noteworthy that only one of our dogs had a half life for diphenylhydantoin comparable to the average value of 7.3 hours in the dogs of CUCINELL *et al.* (1963), whereas our phenobarbital values agreed well with those of REMMER & SIEGERT (1962) However CUCINELL *et al* injected the very high dose of 50mg/kg diphenylhydantoin in order

to determine the half-life of the drug, and such doses might have a depressant effect on the metabolizing enzymes.[1])

It is thus conceivable that results in a rather nonhomogenous dog population can be at variance with those of inbred strains of small rodents where, for example, the shortening of the hexobarbital sleeping time by pretreatment with phenobarbital can be reproduced with the accuracy of a chemical reaction. Hence, the above mentioned sample of dogs with unknown history should come nearest to the situation encountered in the clinical patient. This is characterized by a widely divergent history of drug use and abuse as well as of occupational contacts with foreign compounds as for example pesticides, halogenated hydrocarbons, dyes and so on, all of which have a certain potential influence on the rate of drug metabolism. When such people are put on antiepileptic therapy with, for example, phenobarbital and diphenylhydantoin, they may well behave "atypically", i.e. not react in the way found in small animal experiments, since their rate of drug metabolism is already in a state of stimulation or inhibition. One should also realize that patients treated with phenobarbital and diphenylhydantoin for epilepsy will intercurrently be treated with other drugs for secondary conditions. Again these other drugs may have some influence on drug-metabolizing enzymes and in this way influence the rate of metabolic breakdown of the anticonvulsants. Recently some examples of such interactions with the metabolic fate of diphenylhydantoin have been described (MOLHOLM HANSEN *et al.* 1966, VENDELIN OLESEN 1966, SOLOMON & SCHROGIE 1967).

The above mentioned conditions may give rise to widely diverging "drug-interactions" in patients who are usually regarded as constituting a homogenous material on the basis of clinical diagnosis and therapy. In fact, the reaction of a single patient to combined therapy must be considered as unpredictable. This means that the concentrations of the drugs used must be followed during the treatment, so that both toxic effects and insufficient control of the disease can be avoided. Such a laboratory control seems particularly important in epileptic patients where even serum and tissue concentrations have to be maintained over long periods.

An interesting finding in our experiments is that treatment with diphenylhydantoin apparently also seems to have an effect on the concentrations reached in the course of a simultaneous treatment with pheno-

1) Note added in proof. Recently DAYTON *et al.* (J. Pharmacol. exp. Ther. 158, 305 1967) have shown that the decline of drug plasma levels in the dog really is dose-dependant. Using doses [illegible] 70 mg/kg diphenylhydantoin only they determined half-lives between 1.5 and 2.8 hours which corresponds well to the values in our study. With this dose the decline in half-lives after pretreatment with phenobarbital was much less pronounced than in the previous study of these authors (CUCINELL *et al.* 1963).

barbital (fig. 4). These were lower in dogs treated with both drugs but showed a rising tendency so that the difference between both groups disappeared after the first month. Without concurrent biochemical data no explanation for the phenomenon can be given at present. In this connection it must be realized that the dog metabolizes diphenylhydantoin 3–10 times faster than man where BUTLER (1957) found an average half life of 24 hours. It was thus impossible to maintain even serum concentrations of this drug, with the doses used. We decided, however, not to give higher doses, since these too might give rise to criticism regarding the comparability to the conditions in the patient.

Summary

In dogs treated with phenobarbital and/or diphenylhydantoin for a period of 30 days, the mutual interactions on the rate of metabolic inactivation of both drugs were studied. Apart from the well-known fact that phenobarbital can accelerate its own metabolism as well as that of diphenylhydantoin, an "atypical" behavior was observed in a considerable number of the animals. Thus, the half-lives were not altered by the treatment or even prolonged after it. A possible reason for this, i.e. contacts with other foreign compounds in the period before the experiments, is discussed; its relevance for clinical therapy is pointed out. Drug interactions in the single patient treated with a combination of phenobarbital and diphenylhydantoin seem rather unpredictable and a laboratory control of serum concentrations of both drugs should be carried out in order to avoid toxic effects and insufficient control of the disease.

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5-Hydroxyindoleacetic Acid in Cerebrospinal Fluid after Administration of 5-Hydroxytryptophan I

By

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(Received March 4 1968)

Several investigators have reported that the levels of acid monoamine metabolites are altered in some diseases both in the brain and in the cerebrospinal fluid (CSF) (Sharman 1960 Ashcroft & Sharman 1960 Andersson & Roos 1965 Gottfries, Rosengren A. M. & Rosengren, E. 1965 Andersson & Roos 1966 Ashcroft Crawford Eccleston Sharman, MacDougall, Stanton & Binns 1966 Dencker, Malm, Roos & Werdinius 1966 Johansson & Roos 1967) Changes in the levels of these metabolites in the brain reflect changes in the metabolism of the corresponding amines. The general purpose of the present investigation was to determine the possible correlations between monoamine metabolism in the brain and the levels of the corresponding acid monoamine metabolites in the CSF In particular the levels of 5-hydroxyindole acetic acid (5-HIAA) in the CSF were determined after administration of the precursor 5-hydroxytryptophan (5-HTP).

Methods

Dogs weighing 4–15 kg were kept under light pentobarbital anesthesia induced by intravenous or intraperitoneal injection of 25 mg/kg pentobarbital sodium (Nembutal ®) and supplemented by small repeated doses of the barbiturate. The level of narcosis was kept light to avoid interfering with normal spontaneous breathing. The body temperature was monitored on "Tele-thermometer" (Yellow Springs Instrument Co., Inc., Ohio U.S.A.) and kept normal by placing the animals on an electrically heated operating table. Percutaneous cisternal puncture was performed with a hypodermic needle 1.2 mm in diameter and samples (about 2 ml) were taken from the cisterna magna without allowing intracranial entrance of air Cerebrospinal fluid was withdrawn at regular intervals up to 24 hours. Attempts were made to avoid contaminating the samples with blood. However

samples containing traces of blood were centrifuged immediately and the clear supernatant was deep frozen. Samples with haemolysis were discarded.

Previous to the actual investigation, preliminary experiments with *sampling of CSF* were performed by continuous drip with a needle placed in the cisterna magna, with the dog lying in a horizontal position.

5-HTP (25 mg/kg) and in one experiment, 5-HIAA (1 mg/kg) were dissolved in saline and then injected slowly into a brachial vein. 5-Hydroxytryptamine (5-HT), 5-HIAA and the total 5-hydroxyindoles were determined according to previously published methods (ASHCROFT & SHARMAN 1960 ROOS 1963 ANDÉN & MAGNUSSON 1967). We did not observe any difference between determinations made on samples deep frozen (−20°) for 4–5 days and fresh ones.

Results

The mean cisternal level of 5-HIAA in 24 normal dogs aged between 3 months and 5 years, was 0.07 ± 0.005 μg/ml.

During a control test, the samples of CSF were taken every hour for eight hours and no significant change occurred in the values of 5-HIAA. On the other hand (with the method used before the actual investigation) when samples were taken during *continuous* drip from a needle in the cisterna magna and when the CSF was pooled during one to three-hour periods, the values increased about five times. The amount of CSF collected in those periods was 1.99 ± 0.04 ml/hr thus showing neither an increase nor a decrease in the outflow of CSF during the period of collection.

Injection of 5-HIAA (1 mg/kg i.v.) did not show any increase in the level of the acid in the CSF Half an hour after the injection of 5-HTP (25 mg/kg) the value of 5-HIAA had already risen to 2–3 times the initial

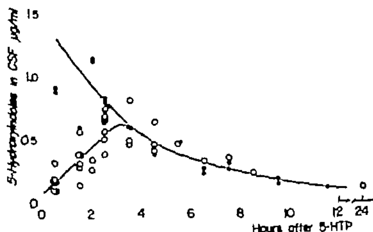


Fig. 1 Effect of intravenous injection of 5-hydroxytryptophan on the levels of total 5-hydroxyindoles (solid circles) and 5-hydroxyindoleacetic acid (open circles).

value, reaching a peak about 3½ hours after the injection (fig. 1) Separate determinations of 5-HIAA, 5-HT and total 5-OH indoles in the CSF were also made. The results show that 5-OH indoles increased in the CSF The value of 5-HIAA one and a half hour after the the injection of 5-HTP was about 30 / of the total concentration of 5-hydroxyindoles At this time no 5-HT could be detected. Three hours after the injection almost all of the 5-hydroxyindoles consisted of 5-HIAA. At this time, in only one experiment could we find any 5-HT (0 03 µg/ml), but 2 hours later the concentration fell to zero again.

Discussion

During the control test, when samples of CSF were taken every hour during nine hours of anesthesia, there were no changes in the values of 5-HIAA. When a sample was taken immediately after the induction of anesthesia and a fresh sample taken five hours later with the dog still under anesthesia, no alteration in the metabolite could be found. These findings imply that the anesthesia by itself has no effect on the level of the metabolite. In contrast to the results obtained after continuous collection, repeated sampling of CSF do not appear to alter the normal levels of 5-HIAA It has been reported that the outflow of CSF in a dog weighing 10 kg is about 0 03 ml per minute (Greenberg, Aird Boelter, Campbell, Cohn & Murayama 1943), which corresponds very well with our findings of 0 033 ml/min.

There are many possible explanations for the increase of 5-HIAA during the continuous collection of CSF described above. Thus the method with an open needle allows the entrance of air into the surbarachnoidal space, which might irritate the meninges. It also disturbs normal contact between the cerebrospinal fluid and the ependymal linings of the ventric ular system and hence the intracranial pressure pattern will be changed. Finally the ventricular volume of cerebrospinal fluid is small as compared with the subarachnoidal volume. The total volume in man is given as 140 ml and of this, there is only about 23 ml in the ventricles (Last & Tompsett 1953) the major part of CSF seems to be produced in the ventricles (Davson 1967). Thus it seems probable that with a free flow of cerebrospinal fluid through the needle, the portion of ventricular cerebrospinal fluid in the sample might be higher The concentration of 5-HIAA has previously been shown to be higher in ventricular CSF as compared with cisternal CSF (Guldberg Ashcroft & Crawford 1966 Andersson 1968).

We did not observe any difference between young and adult dogs. This is in agreement with the observations of Tissari & Pekkarinen (1966)

investigating 5-HIAA in the brain of the developing rat, rabbit and guineapig. A similar observation was reported by SHARMAN (1963) who found that the content of homovanillic acid, the final metabolite of dopamine, in the caudate nucleus rises very rapidly in lambs as early as 4 days after birth thus reaching half the level observed in adult sheep

It was not possible to detect any appreciable amounts of 5-HT in the cerebrospinal fluid although the precursor was injected in high dosage (25 mg/kg) This was expected, as the increase of 5-HIAA in the CSF can be explained by the conversion of brain 5-HT to 5-HIAA, which passes out in the CSF

It is possible that some of the injected 5-HTP penetrates directly from the blood into the CSF In order to investigate this possibility we made separate determinations of 5-HIAA, 5-HT and total 5-hydroxyindoles in the CSF This showed that 5-OH indoles penetrate into the CSF and that the value of 5-HIAA one and a half hour after the injection of 5-HTP is about 30 % of the total 5-hydroxyindole concentration. About two hours later almost all of the 5-hydroxyindoles is 5-HIAA This is in favour of a very rapid brain uptake of 5-OH indoles from the CSF

The highest concentration of 5-HIAA in the brain of the dog following an intravenous injection of 5-HTP seems to occur after approximately 3-4 hours (ANDERSSON & ROOS, unpubl.) The findings in cisternal CSF of a maximal concentration of 5-HIAA about 3½ hours after the injection of the precursor can in our opinion be satisfactorily correlated with these observations on brain tissue. They also support the conclusion that the elimination of acid metabolites from the brain to CSF is rapid and that determinations of 5-HIAA in the CSF could give a good estimate of the changes occurring in the metabolism of 5-HT in the brain.

Summary

5-Hydroxytryptophan, the precursor of 5-hydroxytryptamine, was given intravenously to dogs. Following injection the concentration of 5-hydroxyindoleacetic acid in the cerebrospinal fluid from the cisterna magna was determined at various intervals. The relationship between the increase of 5-hydroxyindoleacetic acid in the brain and the cerebrospinal fluid is discussed.

Acknowledgements

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The Spectrofluorimetric Determination of Phenothiazine Drugs in Blood Serum

By

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(Received F bruary 24, 1968)

Due to a lack of suitable techniques and the relatively low values likely to be found, there is little information available on blood serum values which might be encountered in cases of phenothiazine drug overdosage. RAGLAND *et al* (1964 & 1965) have described a spectrofluorimetric method for the determination of phenothiazine drugs (unchanged) in blood serum. Phenothiazines are extracted from an alkaline solution with n-heptane. The extracted phenothiazine is transferred to 50 / aqueous acetic acid and then treated with hydrogen peroxide By this procedure, sulphoxides are produced which are more intensely fluorescent than the original substances. Determinations are then completed in a spectrofluorimeter using optimum activation and fluorescent wave lengths.

In the present paper the reliability and use of the method are described.

Procedure

Reagents

1. n-heptane.
2. 50 % (/v) acetic acid.
3. hydrogen peroxide 100 volume.
4. normal human serum this should show zero values with the bove procedure. The values obtained are usually slightly lower than water blank.

Apparatus

Aminco-Bowman Spectrofluorimeter fitted with Hanovia H16-992 Xenon Arc Lamp and with Slit Arrangement No. 4 and Sensitivity 25.

Method

Into a 60 ml glass stoppered centrifuge tube are put 5 ml of serum, 1 ml of 10N sodium hydroxide and 40 ml of n-heptane. The mixture is shaken vigorously for 2 minutes and then centrifuged, 35 ml of the heptane extract are transferred to a separating funnel, 5 ml of 50 % acetic acid added and the mixture shaken vigorously for 2 minutes. The acid layer is separated and retained.

Initially the acid extract is examined in the fluorimeter a described below

4 ml of the acid extract is transferred to a test tube and 0.8 ml of hydrogen peroxide (100 volume) added. The tube is placed in a boiling water bath for 10 minutes. After cooling, the mixture is examined in the spectrofluorimeter using appropriate activatio and fluorescent wave length settings.

Blank· A blank is prepared, using 5 ml of normal serum and the complete procedure carried out.

Standards 2.5, 5 and 10 µg of the phenothiazine under investigation or suspected and contained in 5 ml of normal serum are prepared and the complete procedure carried out.

Table 1

Spectrofluorimetry

The activation and fluorescence wavelengths (maximal) in millimicrons of the sulphoxides of the f llowing phenothiazine drugs

Substance	Activation λ	Fluorescence λ
Chlorpromazine	340	385
Thioridazine	365	440
Trifluoperazine	350	405
Perphenazine	345	380
Promazine	340	375
Pipamazine	340	380
Allmemazin	340	380
Promethazine	340	380
Fluphenazine	350	410
Methotrimeprazine	340	380
Triflupromazine	350	410
Thiopropazate	340	380
Methdilazine	340	380
Prochlorperazine	340	380

NOTES

1 Pipamazine cannot be extracted from serum by n-heptane.

2. The sulphoxides of promazine, promethazine, triflupromazine, methdilazine and Allmemazin are extracted from alkaline solution by n-heptane, whereas the sulphoxides of the other phenothiazines are not so extracted.

3. When the identity of the phenothiazine is in doubt, examinations are carried out over the following wavelengths activation/fluorescence 340/380 350/410; 360/440 and 370/475

Table 2

The relative fluorescence of some phenothiazine drugs following treatment with hydrogen peroxide and heat. Fluorescence is measured under optimum conditions for the substance under examination. Thioridazine is taken as 100 for standardisation.

Thioridazine	100
Promazine	32
Trifluoperazine	24
Perphenazine	20
Chlorpromazine	15
Prochlorperazine	7

Results

In table 1 the optimum activation and fluorescent wavelengths used in the spectrofluorimetric determination of 14 phenothiazine drugs are shown.

Under optimum conditions, the sulphoxides of various phenothiazine drugs exhibit marked differences in fluorescence intensity This is shown in table 2.

Results obtained from patients admitted to hospital with symptoms of phenothiazine drug overdosage are shown in table 3 None was fatal.

Discussion

It is obvious that using a single extraction technique recoveries cannot be expected to be optimal. This technique has been adopted in preference to multiple extractions since the latter would result in unduly dilute solutions thus making it difficult to obtain a final evaluation Recoveries above the range of standards used are linear but are lower than when water is used in place of serum The same recoveries are obtained when internal standards are used as when standards are prepared from normal serum. The lower recoveries obtained from serum as compared with water is undoubtedly due to protein binding. Treatment with hot mineral acid has been used by some investigators but this results in considerable loss of free phenothiazine, especially if the concentrations are low

The optimum activation and fluorescent wavelengths for the phenothiazine under investigation should also be used during fluorimetric

Table 3

Phenothiazine drug levels (µg/100 ml serum) in patients admitted to hospital with symptoms of overdosage (non-fatal).

Case No	Phenothiazine	µg/100 ml serum
1	Chlorpromazine	75
2		160
3		125
4		160
5		110
6	Trifluoperazine	115
7		170
8		310
9		210
10	Thioridazine	270
11		1180
12		240
13		245
14		480
15		360
16	Promazine	175
17	Prochlorperazine	115
18		125
19		140
20		160

examination. These show marked variation (table 1) and also the intensity of fluorescence/µg even under optimum conditions (table 2) If the identity of the phenothiazine has not been fully established then a range of activation/fluoresence wavelengths are used (table 1) Urine appears to be the most suitable material for determining the identity of the drug and also whether more than one phenothiazine drug is involved (TOPMSETT 1968).

The sulphoxides of some of the phenothiazine drugs (table 1) can also be recovered by this procedure. Their presence may be determined by fluorimetric examination before hydrogen peroxide treatment. If sulphoxide evaluation is to be included then standards must be prepared in the same way as for the unchanged phenothiazines.

Normal serum yields zero values, in fact they are slightly lower than the water blanks. Interference by non-phenothiazine substances has not been observed.

Under emergency conditions i.e when phenothiazine drug overdosage is suspected, the procedure is important in that the presence or otherwise of a phenothiazine drug in the blood serum can be readily established. This is particularly important since under such conditions, urine is not always immediately available.

Summary

A procedure for the determination of phenothiazine drugs in an unchanged state in blood serum is described and discussed.

Values obtained in patients suspected of non-fatal overdosage and admitted to hospital are reported.

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Acta pharmacol et toxicol 1968, 26, 303–315

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The Detection and Determination of Phenothiazine Drugs in Urine

By

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(Received February 24, 1968)

The purpose of this investigation was to determine the best method for the assessment of the presence, identity and determination of phenothiazine drugs in urine for clinical purposes.

This laboratory is closely associated with the work of the Poisoning Treatment Centre at the Edinburgh Royal Infirmary Scotland It is customary for the laboratory to receive gastric aspirate and lavage, blood (withdrawn on admission) and urine (collected for at least 8 hours) from cases of suspected poisoning. Examination of gastric aspirate and blood has provided some useful data. Urine however has provided sufficient material to carry out more fhuller investigations including identification.

After ingestion, phenothiazine drugs may undergo extensive metabolism. Much work has been carried out on the metabolism of the phenothiazine drugs, but in spite of this, the picture is very incomplete. This subject has recently been reviewed by STOLMAN & STEWART (1965).

The present investigations have been confined to a study of techniques concerned with the detection, determination and identification of unchanged phenothiazine drugs and their sulphoxides. 14 different phenothiazine drugs have been used for reference purposes (see tables).

Techniques

U V examinations

These were carried out in U V recording spectrophotometer (Unicam S.P 800) over the range 200 to 340 millimicrons. Examinations were made against an appropriate blan

Spectrofluorimetry

Such examinations were made by means of a Aminco-Bowman Spectrofluorimeter the appropriate activation and fluorescence wavelengths being used (TOMPSETT 1968).

Thin layer chromatography

Eastman Chromagram Sheets, 20 × 20 cm (Type K301R – silica gel with fluorescent indicator) were used inconjunction with the Eastman Chromogram Developing Apparatus.

Gas/liquid chromatography

Pye Series 104 Gas Chromatograph fitted with Dual Flame Ionisatio Detector
Column – stationary phase – 1 % methylsilicone grease (SE 30).
Operating temperature – 225

Materials

The phenothiazines used were obtained from the appropriate manufacturers.

The sulphoxides were prepared from the appropriate phenothiazine by the method of KOFOED *et al.* (1966).

Extraction techniques

A. A mixture of 25 ml of urine 25 ml of water and 1 g of sodium hydrogen carbonate is placed in a 500 ml flask attached to a water cooled condenser (all glass apparatus) The mixture is heated to boiling and the distillate collected until a volume of 30 ml has been obtained. 3 ml of 10 N hydrochloric acid are added and the mixture examined in a UV recording spectrophotometer (distillate A).

The total distillate, now acid, is diluted to 50 ml with water and the complete distillation and UV examination procedures repeated (distillate B)

This is a modification of the procedure described by GOLDBAUM & DOMANSKI (1965)

B 10 ml of urine are introduced into a 50 ml glass stoppered measuring

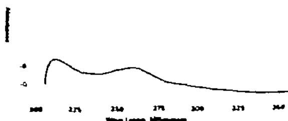


Fig. 1 Normal Urine Extraction Procedure A.

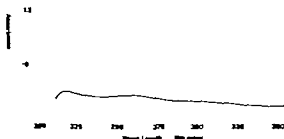


Fig. 2. Normal Urine Extraction Procedure B.

cylinder followed by 1 ml of 10 N sodium hydroxide and 40 ml of chloroform The mixture is shaken vigorously for 2 minutes. After separation, the aqueous phase is removed and discarded. Anhydrous sodium sulphate is added to bring about dehydration, after which it is filtered. 30 ml of the chloroform extract are shaken vigorously for 2 minutes with 10 ml of N sulphuric acid. An aliquot of the acid extract is separated and examined in a UV recording spectrophotometer against an appropriate blank.

In some cases, N hydrochloric acid is used for the final extraction.

C. This is carried out in exactly the same manner as B except that the chloroform extract is evaporated to dryness and the residue dissolved in 10 ml of N hydrochloric acid

D This is carried out in the same manner as B and C except that

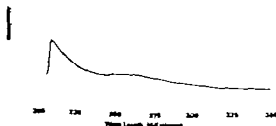


Fig. 3. Normal Urine Extraction Procedure C.

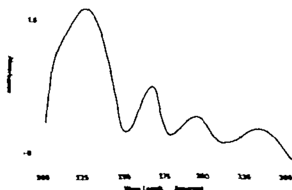


Fig. 8. Promazine Sulphoxide. 25 michrograms/ml. N Hydrochloric Acid.

Results – extraction procedures

The results of the extraction procedures are shown in tables 1 2, 3 and 4

Table 1

Recoveries from urine.

Phenothiazine drugs recoverable by distillation (technique A)

Trifluoperazine	Promethazine	Chlorpromazine
Pipamazine	Trifl promazine	Allmemazine
Promazine	Methdilazine	Methotrimeprazine

Recovery 50–70 / depending on nature of substance

Phenothiazine drugs NOT recoverable by distillation

Fluphenazine	Thioridazine	Perphenazine
Thiopropazate	Prochloroperazine	Propiomazine

Sulphoxides f all the above phenothiazine drugs

Other basic drugs recoverable by distillatio (technique A) and recognisable by their UV spectra

Pyridine	Pethidine (Meperidine)
Amphetamine	Diphenhydramine*)
Methamphetamine	Amitriptyline
Dextropropoxiphen	Imipramine
Tigloidin (Tiglysen)	Diphenylpyraline)
Xylocaine	Phenindamine
β-Phenylethylamine	Preludi (Phenmetrazine)
Tranylcypromine	Nicotine
Orphenadrine)	Diethylpropion (Amfepramo)
nor Triptyline	Benzphetamine
Methadone	Mepyramine
Chlorcyclizine	Ephedrine
N Methylephedrine	Pipradrol
Benzhexol	

) Also recovered i distillate B

Neutral drugs and substances recoverable by distillation (technique A) and recognizable by their UV spectra

Benzene	Acetone	Ethchlorvynol
Toluol	Paraldehyde	

These substances are also recovered in distillate B.

The UV spectrum of ethchlorvynol is changed as the result of the second distillation

Table 2

Extraction technique B.

1 The following phenothiazines are recoverable in the 10 ml. f. N sulphuric acid extract.

Trifluoperazine	Promethazine	Chlorpromazine
Pipamazine	Triflupromazine	Alimemazin
Promazine	Methdilazine	
Fluphenazine	Thioridazine	Perphenazin
Thiopropazate	Prochloroperazine	Propiomazine

The following phenothiazines are recoverable when chloroform solutions are extracted with N hydrochloric acid

Fluphenazine	Perphenazine	Prochloroperazine
Trifluoperazine	Thiopropazate	

The other phenothiazines are not extracted from chloroform solution by N hydrochloric acid.

2. The sulphoxides of the following phenothiazines are recoverable in the 10 ml. of N. sulphuric acid extraction.

Thioridazine*)	Promethazine*)	Chlorpromazine)
Pipamazine*)	Triflupromazine*)	Alimemazin *)
Promazine)	Methdilazine)	

The sulphoxides of the following phenothiazines are not recoverable in the 10 ml. of the N. sulphuric acid extractant.

Fluphenazine	Perphenazine	Prochloroperazine
Trifluoperazine	Thiopropazate	Propiomazine

*) Also recovered when N. hydrochloric acid is used for extraction.
*) not recovered when N hydrochloric acid is used for extraction.

Table 3

Extraction technique D.

1. The following phenothiazines are extractable by heptane.

Trifluoperazine	Promethazine	Chlorpromazine
Promazine	Triflupromazine	Alimemazin
Fluphenazine	Methdilazine	Perphenazine
Thiopropazate	Thioridazine	
Pipamazine	Prochloroperazine	

2. The sulphoxides of the following phenothiazines are extractable by heptane.

Promazine	Promethazine	Triflupromazine
Methdilazine	Alimemazin	

The sulphoxides of the following phenothiazines are not extractable by heptane

Fluphenazine	Thioridazine	Pipamazine
Perphenazine	Thiopropazate	Prochlorperazine
Propiomazine	Trifluoperazine	

Table 4

Extraction technique E.

The sulphoxides of the following phenothiazines are not recoverable by means of extraction techniques B, C and D but are recoverable by means of extraction technique E.

Thiopropazate	Perphenazine	Fluphenazine
Prochlorperazine	Trifluoperazine	Propiomazine

Phenothiazines By the use of the distillation procedure, the phenothiazines can be divided into two groups – volatile and non-volatile. In the case of the volatile phenothiazines, recoveries were of the order of 50–70 %, depending on the nature of the phenothiazine under investigation. Recoveries of 250 to 2000 μg of 'volatile' phenothiazine added to water or urine had a linear relationship. Quantitative results can be obtained by the use of this technique. Recoveries from water and urine were identical.

As indicated in table 1 certain other basic drugs and some neutral substances may be recovered and their presence indicated by the same procedure. The neutral volatile substances are recoverable in distillate B. With the exception of diphenhydramine, orphenadrine and diphenylpyraline, volatile basic substances are not recoverable in distillate B.

Distillation was found to have no effect on the UV spectrum as compared with that of the original substance.

All the free phenothiazines examined could be recovered when extraction procedure B was used and N sulphuric acid was used as the final extraction. When 250 to 1000 μg quantities were examined, the recoveries were linear indicating that quantitative results can be obtained by the use of the procedure. Recoveries from urine and water were identical.

Only some of the phenothiazine substances could be recovered from chloroform solution by using N hydrochloric acid in the final extraction. This phenomenon can be used as a means of differentiation. McBay & Algeri (1963) have shown that many phenothiazines form complexes

with chloride ions, and that these complexes are very soluble in chloroform. It is not possible to follow an extraction with N hydrochloric acid with one using N sulphuric acid.

All the phenothiazine substances examined are recoverable by extraction with n-heptane. The phenothiazine can be recovered from the heptane extract, either by removal of the solvent by evaporation or by extraction with N sulphuric acid. This is a quantitative procedure.

The remainder of the extraction procedures can be used but is not required under the circumstances

Phenothiazine sulphoxides None of the sulphoxides examined were recoverable by distillation.

The sulphoxides showed less readiness to be separated by organic solvents than the unchanged phenothiazines. Such data have proved useful in effecting certain separations. Thioridazine can be separated from the sulphoxide by the use of n-heptane.

The six phenothiazine sulphoxides referred to in table 4 cannot be recovered by the use of extraction procedures B C and D Recovery can be obtained by the use of extraction procedure E, quantitative recoveries being made over the range 250 to 1000 µg.

The recovery of volatile basic drugs

Following the identification of the presence of volatile basic drugs in distillates by UV examination, their recovery in solid or concentrated form may be required for further examination.

With the exception of diphenhydramine, orphenadrine and diphenylpyraline, the basic drugs can be recovered by evaporation of the distillate following the addition of excess of hydrochloric acid. Such residues are bulky due to the presence of such substances as ammonium salts.

Extraction with ether following the addition of alkali generally effects separation from most of the unwanted material. Ether extracts may be allowed to evaporate at room temperature, followed by dehydration in a cabinet containing silica gel. Methamphetamine, amphetamine, β-phenylethylamine and nicotine are lost whereas other volatile bases are completely recovered. The above four bases can be recovered by the addition of hydrochloric acid to the ether extract and evaporation to dryness in an all glass vacuum still. These bases are recovered as the hydrochlorides. Other bases are recovered in the free state.

Spectrofluorimetric assay

The present spectrofluorimetric examination of urine distillates and untreated urine has proved useful in establishing the presence of a pheno-

thiazine. Only approximate identity can be established by the use of this technique.

Gas/liquid chromatography

The results reported by KOFOED *et al* (1966) have been confirmed but the technique is limited since many of the phenothiazines and especially of the sulphoxides are not volatile.

Thin layer chromatography

KOFOED *et al* (1966) have indicated that phenothiazine substances are largely converted into the sulphoxides during the development on the plates. It would appear that it is preferable to use the sulphoxides, rather than the unchanged substances, to obtain identification by means of this technique and particularly since the former represents a major fraction in urine It is relatively simple to separate the sulphoxides from urine.

The sulphoxides derived from 14 phenothiazine drugs have been examined by the technique described by KOFOED *et al* The developing solvent was – ammonium acetate, 1.5 g/water 10 ml/methanol, 40 ml.

Visualisation was made by (1) examination under UV light (250 millimicrons) and (2) the application of the Dragendorff reagent. Under UV light, all the phenothiazine sulphoxides appeared as dark spots with the exception of thioridazine sulphoxide which exhibited a bright blue fluorescence.

The range of R_f values were similar to those reported by KOFOED *et al*

Colour reactions

FORREST *et al* (1961) have summarised their findings on a number of very useful colour reactions that have been developed over a number of years for the detection and semi-quantitative determination of phenothiazine drugs and some of their metabolites in urine Positive reactions appear to be given by the unchanged phenothiazine drugs and hence certain metabolites still largely undefined and not considered in this paper but not by the sulphoxides. Unfortunately in only a few cases can the identity of the phenothiazine be established or even partially established by the use of these colour reactions.

NEVE (1961) has described a quantitative colour reaction for the detection and determination of thioridazine and certain metabolites, largely undefined, in urine An extract of urine is treated with sulphuric acid and the blue colour that develops assessed after standing for 24 hours. The author has modified the test which can now be applied directly to

Table 5

The urinary excretion of unchanged phenothiazine and phenothiazine sulphoxide in cases admitted to hospital with symptoms of overdosage.
The results are expressed in mg/100 ml urine

Case No.	Nature of pehnothiazine	Unchanged phenothiazine	Phenothiazine sulphoxide
1	Trifluoperazine	2.5	6.8
2		1.9	4.3
3		1 7	5 1
4	Chlorpromazine	1.9	18.6
5		2.2	21.8
6		3.5	30.6
7		2.8	18 4
8		2.6	24.8
9		3 1	28.8
10		3 6	36.2
11		4.8	40.8
12	Promazine	3.6	30.2
13		2.3	19 6
14		1.8	25.2
15	Prochloroperazin	1.8	18.2

urine. 3 ml of 10 N hydrochloric acid are added to 1 ml of urine and the blue colour that develops assessed against standards after standing at room temperature for 24 hours. The reaction which appears to be specific for thioridazine is given by the unchanged substance and by certain ill defined metabolites, but not by the sulphoxide.

Poisoning cases

Results obtained from the examination of urine from cases admitted to hospital with symptoms of phenothiazine drug overdosage are shown in table 5 No cases were fatal.

Discussion

Patients are frequently admitted to hospital with symptoms suggestive of drug overdosage but even when verified few have proved fatal.

The information obtained from various sources, including the patient, has often proved insufficient and sometimes contradictory Although gastric aspirate and lavage, blood and urine are all usually available,

examination of urine has proved to be most profitable. Gastric aspirates have only appeared to be of value when large quantities of drugs have been consumed e.g. in suicidal attempts, but these have been in the minority. There is a lack of information concerning blood levels of many drugs following therapeutic dosage and overdosage. It has become the policy in this laboratory to apply screening methods only to urine after which blood levels are determined, provided that a suitable method is available. Unfortunately suitable methods for the determination of the blood levels of some drugs are not yet available. The screening of urine has resulted in a less likely chance of a drug being missed. Concentrations are usually much higher in urine than in blood.

The procedures described form part of a scheme for the rapid screening of urine for the presence of ingested drugs; the whole scheme is capable of completion within 1 hour. The objective of the scheme is not only to indicate the possible presence of certain drugs but the definite absence of others. The coverage is large but not entirely complete. Having established the presence of a drug or group of drugs, one can then apply more specific techniques in order to establish identity.

The majority of the unchanged forms of the phenothiazine drugs likely to be encountered in urine, are recoverable in the distillation procedure and are readily recognised by UV spectrophotometry. Of the metabolites, the sulphoxides are the most readily recovered and identified. Individually this group show differences in their ability to be recovered by extraction with organic solvents. Unless great care is exercised in the selection of the extraction procedure, certain of the phenothiazine sulphoxides e.g. that of perphenazine, could be easily missed.

For identification purposes, gas/liquid chromatography appears to have its limitations. Identification appears to be more readily achieved by the use of thin layer chromatography.

Summary

Methods for the identification and determination of unchanged phenothiazines and their sulphoxides in urine are described.

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Forrest, I. S., F. M. Forrest & A. S. Mason: Review of Rapid Urine Tests for Phenothiazine and Related Drugs. *Amer. J. Psychiat.* 1961, 118, 300–307.

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bitartrate 5 or 10 μg/ml was used. Five or fifteen minutes after the subcutaneous injections the animals were killed by decapitation. The two pieces of skin were excised and weighed and their total contents of radioactivity determined (^{14}C-tracer substances in Packard Tri-Carb Liquid Scintillation Spectrometer model 3003, ^{131}I-human serum albumin in a scintillation well counter connected to an I.D.L. Scaler 1700). The difference in radioactivity between the two pieces of skin was expressed in per cent of the injected dose ("residual radioactivity per cent") and the rate constants for the period 0–15 minutes for process of the first order were calculated (KETY 1948 & 1949 cf. SECHER HANSEN LANGGÅRD & SCHOU 1968) and correlated to the molecular weight of the substances.

Experimental groups

1) The absorption rate for mannitol, sucrose and inulin dissolved in H_2O with and without hyaluronidase was determined in oestradiol pretreated mice (the results were compared with those of a previous study with non-treated mice (SECHER HANSEN LANGGÅRD & SCHOU 1968).

2) The rate of absorption for mannitol, sucrose and inulin dissolved in 0.9 % NaCl with and without hyaluronidase was determined in normal as well as in oestradiol pretreated mice.

3) The rate of absorption for sucrose molecules in 10 % sucrose solution with and without hyaluronidase was determined in oestradiol pretreated mice. (The results were compared with those from a previous investigation for ormal mice (SECHER HANSEN LANGGÅRD & SCHOU 1967b).

4) The rate of absorption for mannitol, sucrose and albumin dissolved in 0.9 % N Cl with and without *adrenaline- or noradrenaline* was determined in on-oestradiol treated mice.

Results

Fig. 1a shows that in a purely aqueous solution, mannitol molecules disappear faster than sucrose molecules, which in turn disappear faster than inulin molecules. Addition of hyaluronidase causes the three substances to disappear at the same rate. In a semilogarithmic system the results are presented as rectilinear or approximately rectilinear curves (fig. 1b).

In fig. 2 the relation between the molecular weight of the substances and their absorption rate constants are compared in oestradiol pretreated and normal animals. In both groups a decreasing absorption rate constant is found with increasing molecular weight. The rate constants are lowest in the oestradiol pretreated group, but the course of the curves are parallel. Addition of hyalyronidase increases the values, particularly as far as the larger molecules are concerned, so that they become equal for all three substances. The curves for oestradiol pretreated animals and normal animals are still parallel and now also parallel to the abscissa axis, and with the same distance between them as before the addition of hyaluronidase.

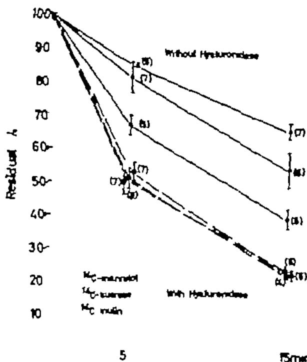


Fig. 1a

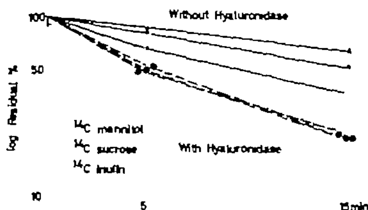


Fig. 1b

Fig. 1a + Fig. 1b. Residual radioactivity per cent 5 and 15 minutes after subcutaneous injections of 80 µl of H_2O containing approximately 0.3 µc of ^{14}C-tracer substances without (upper curves) or with 40 i.u. of hyaluronidase per dose (lower curves).
The standard error of the mean is indicated by vertical lines. Figures in brackets show number of animals. The residual radioactivity per cent is given in a *semilogarithmic system* in fig. 1b.

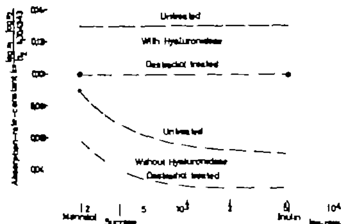


Fig. 2. The absorption rate constant (first order process) f the ^{14}C-tracer substances used is shown (ordinate) in relation to the logarithm of the respective molecular weights (abscissa). The bsorption rate constant is calculated on the basis f residual values 15 minutes after subcutaneous injections into the skin of oestradiol pretreated and normal mice of 80 µl of distilled water containing approximately 0.3 µc of the ^{14}C-tracer substances without (lower curves) or with the addition of 40 I.u. of hyaluronidase to the injected volume (upper curves).

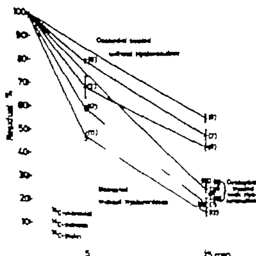


Fig. 3. Residual radioactivity per cent 5 and 15 minutes after subcutaneous injections of 80 µl of 0.9 % NaCl containing approximately 0.3 µc of ^{14}C-tracer substances without or with 40 I.u. of hyaluronidase per dose.

The standard error of the mean is indicated by vertical lines.

Figures in brackets show number of animals.

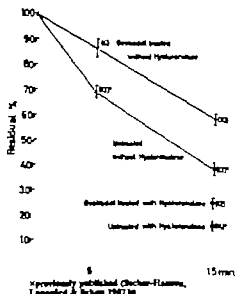


Fig. 4 Residual radioactivity 5 and 15 minutes after subcutaneous injections of 80 µl of 10 sucrose containing approximately 0.3 µc of ^{14}C-sucrose without (upper curves) or with 40 i.u. of hyaluronidase (lower curves) per dose into the right of two symmetrical 5.5 cm^2 areas of depilated skin on the back of oestradiol pretreated and normal mice.
The standard error of the mean is indicated by vertical lines.
Figures in brackets show number of animals.

From a 0.9 / NaCl solution injected the mannitol molecules disappear *more slowly* than the larger sucrose molecules (fig. 3) This is observed both after five and fifteen minutes in oestradiol pretreated as well as in normal mice. Furthermore, the mannitol molecules disappear *more slowly* from a 0.9 / NaCl solution rather than from a purely aqueous solution injected (fig. 1a & 3). In contradistinction the sucrose and inulin molecules disappear *more quickly* from a 0.9 / NaCl solution than from water (fig. 1a & 3). In the oestradiol pretreated animals the addition of hyaluronidase increases the rate of disappearance for each of the three tracer substances in such a way as to make them equal.

Following a 10 / sucrose solution injected subcutaneously into oestradiol-treated mice, the radioactive sucrose molecules disappear at the same rate as from pure water (fig. 4) – and thus more slowly than from an injection of a 0.9 / NaCl solution (fig. 1a, 3 & 4) The addition of hyaluronidase caused a faster disappearance rate of sucrose molecules from the injected 10 / sucrose solution so that the absorption rate becomes the same as from water and from 0.9 / NaCl containing hyaluronidase (fig. 4) Adrenaline or noradrenaline delay the disappearance rate of mannitol and sucrose molecules (fig. 5). The effect of these vasoactive

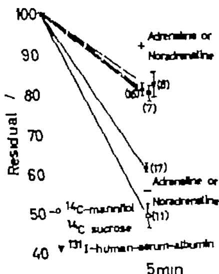


Fig. 5. Residual radioactivity per cent 5 minutes after subcutaneous injections of 80 μl of 0.9 % NaCl containing approximately 0.3 μc of ^{14}C-mannitol or ^{14}C-sucrose without or containing 5–10 μg/ml adrenaline or noradrenaline per dose or approximately 0.5 μc of ^{131}I-human serum albumin containing 5–10 μg/ml adrenaline or noradrenaline per dose, into the right of two symmetrical 5.5 cm² areas of depilated skin on the back of normal mice.
The standard error of the mean is indicated by vertical lines.
Figures in brackets show number of animals.

substances in concentrations of 5 μg/ml did not differ from the effect of concentrations of 10 μg/ml. The residual radioactivity per-centage, 5 minutes after the injections of radioactive albumin without any vasoactive substances, has regrettably not been investigated in parallel experimental series, but this percentage can not be below the residual radioactivity per cent when vasoactive substances are present in the injected volume, as the disappearance-rates of albumin 15 minutes after the injections of standard volumes with and without vasoactive substances are identical. At this time 80 % ± 3.9 (s.e.m. n = 9) remained without any vasoconstrictor substances added, while 84 % ± 1.4 (n = 6) remained after the addition of noradrenaline or adrenaline to the injected volume (unpublished). Furthermore experiments with the same strain of mice, but reared at another place, showed that the residual radioactivity per cent of albumine injected without the addition of vasoactive substances was 94 % ± 1.1 (n = 5) 5 minutes after the injection and 85 % ± 1.8 (n = 5) 15 minutes after the injection. Thus the addition of adrenaline or noradrenaline to the injected volume delays the disappearance rate of mannitol and sucrose molecules so that they both are cleared at the same

rate as the albumin molecules. The clearance rate of albumin is independent of addition of hyaluronidase (SECHER HANSEN 1968).

Discussion

The results in this paper confirm previous investigations on the significance of the gel structure of the ground substance for the absorption of compounds with different molecular weights. The absorption rate decreases with increasing molecular weight, whereas this can be abolished by addition of hyaluronidase (fig. 1).

It is further observed that the absorption takes place at a lower rate in oestradiol pretreated animals than in normal animals (fig. 2), presumably because of the higher content of hyaluronic acid in the skin of the pretreated animals. Investigations *in vitro* (LAURENT & PIETRUSZKIEWICZ 1961) have shown a directly proportional relation to the sedimentation rate for a given molecule in a hyaluronic acid solution and the concentration of the hyaluronic acid. The results in the present work indicate a certain conformity the rate of absorption of mannitol sucrose and inulin being reduced to approx imately one half in the oestradiol pretreated mice (fig. 2) in which the quantity of ground substance is approximately doubled (HVIDBERG SZPORNY & LANGGÅRD 1963)

In normal animals a quicker rate of absorption is observed in the period 0–5 minutes than in the period 5–15 minutes after the subcutaneous injections, presumably due to an initially greater filtration (SECHER HANSEN LANGGÅRD & SCHOU 1968). In contrast, practically rectilinear absorption curves are obtained in the oestradiol pretreated animals (fig. 1b) in which the clearance of the substances may be assumed to depend to a higher degree on diffusibility

When mannitol, sucrose and inulin are used as tracer substances in an 0 9 / NaCl solution these molecules disappear more slowly in oestradiol pretreated mice than in normal animals (fig. 3) However mannitol and sucrose have here, so to speak, "changed places" so that the sucrose molecules are absorbed more rapidly than the smaller mannitol molecules. As the mannitol molecules moreover disappear more slowly from an 0.9 / NaCl solution than from the aqueous solution – in contradistinction to the sucrose and inulin molecules – the explanation should presumably be sought in conditions related to the absorption of mannitol molecules in the presence of an excess of sodium and/or chloride. The explanation is, however uncertain Perhaps this finding should be considered in conjunction with the fact that in certain respects "physiological saline" – despite the name – is found to be most unphysiological This appears

from previous investigations, where, as far as several parameters are concerned, the 0.9 % NaCl solution has shown conformity with solutions containing hyaluronidase (SECHER HANSEN, LANGGÅRD & SCHOU 1967b). Contrary to this, fig. 4 shows that the absorption of sucrose molecules from a 10 % sucrose solution, as far as all values are concerned, corresponds to the absorption of sucrose from a purely aqueous solution. In this respect a 10 % sucrose solution may be said to be more "physiological" than 0.9 % NaCl.

It was previously found (SECHER HANSEN 1968) that a subcutaneously injected *volume* was cleared at the same rate as radioactive albumin molecules contained in it. As albumin is mainly or exclusively removed by the lymphatics, it was suggested that volumes injected subcutaneously were also removed essentially by the lymphatics. In the present work it is found that addition of vasocontractile substances which are assumed to stop or greatly reduce the local blood flow does *not* influence the disappearance rate of albumin, which confirms the assumption that albumin disappears through the lymphatic vessels in which no contractile elements are found. Moreover, that mannitol as well as sucrose molecules after the addition of adrenaline or noradrenaline into the injected volume, disappear at the same rate as the albumin molecules and thus at the same rate as the injected volume. Considering that the clearance from the tissue of volumes of substances injected subcutaneously takes place partly by filtration (i.e. shifting of quantities of liquid because of differences in hydrostatic pressure), partly by diffusion (i.e. shifting of quantities of substances because of differences in concentrations) it is possible that the filtration can be identified by an increased flow of lymph.

Summary

The rate constants for the subcutaneous absorption of mannitol, sucrose, inulin and albumin have been determined in normal mice and oestradiol pretreated mice, in which the quantity of ground substance is approximately doubled. After injections of the tracer substances in a purely aqueous solution a decreasing rate of absorption was found with increasing molecular weight in both experimental groups, but the differences were abolished when hyaluronidase was added to the injected solutions. In the oestradiol-pretreated animals the absorption took place more slowly than in normal animals, presumably on account of the higher hyaluronic acid concentration. After injections of the tracer substances dissolved in 0.9 % NaCl the mannitol molecules were removed more slowly than the larger sucrose molecules. The addition of adrenaline or

noradrenaline to the injected volume show at a far slower rate, namely at the same rate as albumin the disappearance rate of which was independent of the presence of adrenaline and hyaluronidase, and which furthermore disappeared at the same rate as the liquid surplus injected.

Acknowledgements

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Studies on the Role of the Intestinal Microflora in the Metabolism of Coumarin in Rats

By

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(Received February 14 1968)

The metabolism of coumarin (fig. 1) has been studied by MEAD *et al.* (1958), FURUYA (1958a), BOOTH *et al* (1959) and KAIGHEN & WILLIAMS (1961) These investigations, carried out mainly in the rabbit and rat, showed that coumarin is metabolized principally by hydroxylation or fission of the heterocyclic ring. The latter reaction results mainly in the formation of *o*-hydroxyphenylacetic acid (fig. 1) The findings of FURUYA (1958a) and BOOTH *et al.* (1959) indicated that ring fission also leads to the formation of *o*-coumaric and melilotic acids (fig. 1). However KAIGHEN & WILLIAMS (1961), using [3-^{14}C] coumarin, did not find these two metabolites in the urines of rabbits and rats treated with coumarin.

This discrepancy together with the knowledge that related heterocyclic ring systems such as those found in flavonoid compounds are split by the intestinal microorganisms (BOOTH & WILLIAMS 1963 GRIFFITHS 1964), led us to assume that opening of the coumarin ring to give *o*-coumaric and melilotic acids may take place in the intestinal tract as a result of microbial action. If this is the case, the observed differences in coumarin metabolism may reflect differences in the gastrointestinal microflora of the animals used. The present study was therefore undertaken to investigate the ability of the rat intestinal microflora to metabolize coumarin

Methods

Animals

Male albino rats, weighing from 340–370 g. were used. They were maintained on commercial pellet diet (Felleskjopet, Oslo).

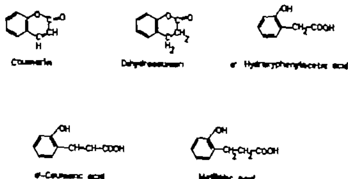


Fig. 1 Structural formulae of coumarin and related compounds.

Compounds

The compounds were obtained from commercial sources and, if necessary were purified before use. They were shown to be chromatographically pure in the solvent systems used.

Incubation with caecal xtracts

The incubation medium and methods used have been described previously (SCHELINE 1968). Coumarin (5 mg) was dissolved in the medium (10 ml) by placing the tubes in a boiling water bath. Controls were similarly prepared except that the caecal extract was omitted.

Animal experiments

The rats were fed a purified diet beginning two days before treatment as described previously (SCHELINE 1968). They had free access to drinking water

Coumarin (100 mg/kg) was given by stomach tube as a fine powder suspended in water containing a small quantity of bile salt or by intraperitoneal injection dissolved in propylene glycol-water (1 1). The urine and faeces were collected separately in containers placed in solid carbon dioxide (SCHELINE & LONGBERG 1965)

Urine samples, after thawing and filteri g, were diluted to 30 ml, djusted to pH 2 ith hydrochloric acid and extracted with three 25 ml portions of ether-ethyl acetate (3 1). The extracts were shaken with three 25 ml portions of 5% sodium bicarbonat solution to remove acidic compounds. These aqueous solutions, after acidifying to pH 2, were re-extracted with ether The ether extracts were dried over nhydrous sodium sulphate, evaporated and the residues dissolved in 1 ml acetone.

Chromatography

The above acetone solutions, together with appropriate standards, were examined by thin-layer chromatography on 0.5 mm thick layers of cellulose. The relevant chromatographic data are shown in table 1 An indication of the relative amounts of the metabolites present in the solutions was obtained by comparing the colour intensities of the spots on the chromatograms.

Table 1

Thin-layer chromatography and colour reactions of coumarin and related compounds.

Compound	Rf value in solvent				Fast blue B salt	Colour with:	
	1	2	3	4		Gibb's reagent	Ultraviolet light 350 nm
Coumarin	1.0	45	96	–	–	–	absorbs
Dihydrocoumarin	1.0	.45	87	98	red	blue	absorbs
o-Hydroxyphenylacetic acid	.37	77	73	64	red	blue	absorbs
o-Coumaric acid	.39	.23	44	64	purple-red	blue	blue-white
Melilotic acid	.66	63	71	84	red	blue	absorbs

diffuse spot.

Solvent
1 benzene-glacial acetic acid-H_2O (6 7:3 upper phase)
2 20 % aqueous potassium chloride-glacial acetic acid (100 1)
3: n-propanol-ammonia 0.88 (7:3)
4 chloroform-glacial acetic acid-H_2O (2 1 1 lower phase)
Cellulose for thin-layer plates
Solvents 1 and 4 Sigmacell Type 19 (Sigma Chemical Co.)
Solvents 2 and 3 MN 300 (Macherey Nagel and Co.)
Spray reagents
Prepared as described previously (Scheline 1966).

Results

Metabolism by the intestinal microflora

Incubation of coumarin anaerobically for 22 hours with the rat caecal extracts, resulted in the formation of a metabolite showing the chromatographic characteristics and colour reactions of melilotic acid. Caeca from 11 rats were used to prepare 18 samples and this compound was consistently observed on the chromatograms. *o*-Coumaric acid and *o*-hydroxyphenylacetic acid were not detected in any of these experiments. Similar results were obtained when rabbit faecal extracts were used. The only other metabolite seen was dihydrocoumarin which was readily separated from coumarin and melilotic acid in solvent 3.

Further confirmation of the identity of the acidic metabolite was obtained by performing a large-scale experiment (48 hour incubation) using medium (800 ml), coumarin (500 mg) and caecum extract (10 ml).

Purification of the ether extract was achieved by chromatography in solvent 3 followed by a solvent of 20 / aqueous potassium chloride. This material was dissolved in 0 1 N HCl and gave an absorption maximum at 270 nm (Beckman DB) and excitation and fluorescence maxima at 274 and 304 nm respectively (Aminco-Bowman Spectrophotofluorometer uncorrected values). These values were identical to those obtained with an authentic sample of melilotic acid.

No melilotic acid was formed in control samples incubated without any caecum extract or with caecum extract which had been heated at 80° for 15 minutes or autoclaved at 121 for 15 minutes. The formation of melilotic acid was not affected when sulphathiazole (100 µg/ml) or mycostatin (35 units/ml) was present in the incubates but was greatly reduced when oxytetracycline (10 µg/ml) or a mixture of neomycin sulphate (25 µg/ml) and bacitracin (1.5 units/ml) was present. Oxytetracycline reduced the amount of melilotic acid formed to approximately 1–5 / of the normal value. Some decrease was evident at a level of 1 µg/ml but the reaction could not be abolished entirely even at a level of 50 µg/ml.

While control samples or samples showing only slight growth will maintain a pH value of 7 4 or slightly below those incubated with caecum extract give values of 6 4–6 6 Hence similar experiments were made using a medium containing a pH 6.4 phosphate buffer Control samples incubated with coumarin but no caecal extract showed only unchanged compound. When the caecal microorganisms were present, melilotic acid was formed although the amount was less than seen with pH 7 4 buffer The reaction resulting in the formation of melilotic acid from coumarin is therefore not a non-specific one due to the pH value of the medium. However when dihydrocoumarin was incubated in medium at either pH 7 4 or 6 6 it underwent extensive spontaneous fission to melilotic acid

Metabolism in rats

The most prominent acidic metabolite in the 24-hour urines following administration of coumarin was *o*-hydroxyphenylacetic acid. The amounts present were similar following oral or intraperitoneal dosage. Very small amounts of *o*-coumaric acid were also detected in these samples.

Melilotic was always detected and following oral coumarin dosage (14 rats), was estimated to be present to the extent of 5–10 / of the amount of *o*-hydroxyphenylacetic acid. The results obtained following injection of coumarin (10 rats) were variable but melilotic acid was always observed In some experiments, the amounts obtained were equal to those found after oral dosage As a rule, however they were lower and ranged from 20–50 /. The amounts observed after injection were not

dependent on biliary excretion, as the results were similar when the common bile duct was tied off and severed before coumarin administration (3 rats). These metabolites were not detected in the urine of control animals (4 rats)

The urines of 2 rats given coumarin orally were chromatographed in solvent 3 before being shaken with sodium bicarbonate solution, in order to determine whether dihydrocoumarin was present. In neither case was this metabolite detected.

The effect of pretreatment of rats for four days with purified diet containing 1 / oxytetracycline or neomycin sulphate was studied The urines of both the antibiotic treated and non-treated rats following intraperitoneal injection of coumarin contained melilotic acid However the amounts detected in the urines of the treated rats (6 animals) were always lower and were estimated to be about 20–50 / of the amounts from the rats not receiving antibiotics (6 animals).

Discussion

The present findings indicate that the rat and rabbit intestinal microflora convert coumarin to melilotic acid. The two most likely routes for this process are ring fission to give *o*-coumaric acid followed by reduction to melilotic acid or reduction to dihydrocoumarin followed by ring fission. It seems unlikely that the first pathway is involved as *o*-coumaric acid was never detected in the coumarin incubates. Although the reduction of *o*-coumaric acid to melilotic acid has been shown to be brought about by the intestinal microflora (SCHELINE 1968) it seems improbable that this reaction would be so extensive that no trace of the intermediate could be found. However it is known that microorganisms can metabolize coumarin to melilotic acid via *o*-coumaric acid. LEVY (1964) demonstrated that *Arthrobacter* sp isolated from soil could bring about this reaction and found both acidic metabolites in the incubation medium. The present results indicate that coumarin is first reduced to dihydrocoumarin which undergoes spontaneous ring fission to give melilotic acid

The fact that full inhibition by antibiotics could not be achieved in the incubation experiments may be relevant to the finding that rats fed a diet containing antibiotic also excreted melilotic acid after coumarin treatment, although the amounts were lower than those obtained when the ordinary purified diet was used. This may indicate that only a partial inhibition of the intestinal microflora which carries out the reaction has occurred However these findings can also be interpreted as indicating that an antibiotic-sensitive part of the conversion takes place in the

intestine as a result of the activity of microorganisms while further conversion which is unaffected by antibiotics may occur in the tissues.

Two differences in the metabolism of coumarin in the rat compared with that in the incubates with intestinal microflora require comment. First, while no *o*-coumaric acid was detected in the incubates, trace amounts of this substance were usually found in the urine. The most likely explanation is that it results from the further metabolism of melilotic acid in the tissues. Oral administration of the latter compound (100 mg/kg) led to the excretion of small amounts of *o*-coumaric acid in the urine (SCHELINE, unpublished results). This reaction has also been reported by FURUYA (1958b) and BOOTH *et al* (1959). The other difference concerns the finding of dihydrocoumarin in the incubates but not the urine. Here it seems likely that reabsorption of this lipophilic substance from the kidney tubule would be adequate to prevent any significant urinary excretion of this metabolite which, after all is formed in only small amounts. However FURUYA (1958a) has reported the presence of dihydrocoumarin in the urine of coumarin-treated rabbits.

Another point dealing with permeability is the finding that intraperitoneal injection of coumarin leads to the excretion of melilotic acid and that this is not dependent on biliary excretion which could conceivably transport some of the coumarin to the intestine. However coumarin is a relatively small molecule having solubility properties which would enable it to diffuse easily into the intestinal lumen. Also the amounts of melilotic acid detected following injection were generally less than those found after oral dosage. Therefore, the conversion of coumarin to melilotic acid after injection can also be explained on the basis of an intestinal reaction although as pointed out above, it is possible that the reaction also occurs in the tissues.

Therefore, the present data do not allow a final decision about the site or sites of formation of melilotic acid following coumarin administration. Although this conversion has been shown to be carried out by the intestinal microflora, other sites cannot be excluded. Nevertheless, it seems most likely that the reaction is not due to the tissues since MEAD *et al* (1958) and KAIGHEN & WILLIAMS (1961) demonstrated the absence of melilotic acid in the urine of coumarin-treated rats and rabbits. It seems most reasonable to assume that the reported differences in coumarin metabolism reflect differences in the capabilities of the intestinal microflora rather than differences in the tissue metabolism of the various groups of rats and rabbits used.

Summary

Coumarin was metabolized by the rat caecal microflora to melilotic acid. The initial step involved reduction to dihydrocoumarin which underwent ring fission to give melilotic acid. No evidence was obtained for the participation of *o*-coumaric acid in the sequence.

Melilotic acid was found to be a urinary metabolite of coumarin in rats. Its origin appears to be intestinal although the possibility that it may also arise in the tissues could not be excluded

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The Metabolism of Drugs and Other Organic Compounds by the Intestinal Microflora

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Studies on the metabolic fate of drugs and foreign organic compounds up to the present have dealt almost exclusively with the changes occurring in the tissues. These reactions include oxidation, reduction hydrolysis and conjugation and have been the subject of a review by GILLETTE (1963). However drug metabolism need not be confined to the tissues, as oral administration of the drug or the excretion of the drug or its metabolites in the bile will bring it into contact with the gastrointestinal microflora, where metabolic alterations may take place.

Studies dealing with the metabolism of drugs by the gastrointestinal microflora have been carried out in only a few instances and much of our present knowledge of the subject comes from incidental observations made in the course of other studies. Increasing numbers of such reports have appeared in the past few years with the result that the possibilities of drug metabolism by the gut flora are becoming more widely recognized. Investigations carried out in this laboratory (SCHELINE & LONGBERG 1965 SCHELINE 1966a & b 1967 & 1968a) demonstrated the importance of the intestinal microflora in the metabolism of a sulphonated azo dye and a number of phenolic acids These results prompted a fuller study of the metabolic capabilities of the microflora The present report deals with the metabolism by the rat caecal microflora, of a number of compounds belonging to some common classes of foreign compounds, drugs or their metabolites.

Methods

Compounds

The following compounds were prepared 4-methylumbelliferone glucuronide (MEAD, SMITH & WILLIAMS 1955) and 8-hydroxyquinaldic acid (IRVING & PINNINGTON 1957). The

other compounds were obtained commercially. They were checked for purity chromatographically and purified if required.

Incubation with caecal extracts

Unless otherwise noted the test substance (5–10 mg) was incubated anaerobically for 22 hours at 37° in a medium containing glucose, yeast extract and peptone in pH 7.4 phosphate buffer as described previously (SCHELINE 1966a). Caeca were obtained from male albino rats maintained on a commercial pellet diet (Felleskjøpet, Oslo). Caecal extracts were prepared by mixing the entire caecal contents with 5 volumes of medium and centrifuging for about 15 seconds at the lowest speed on a small centrifuge to remove debris. One ml of the supernatant was added to the sample before incubation. Controls were similarly prepared except that the caecal extract was omitted.

Extraction of incubates

Following the incubation period the samples were acidified as follows: 1 ml concentrated HCl added to phenyl-β-D-glucoside, hesperidin, rutin, methyl gallate, pyrogallol, (+)-catechin, hesperetin, quercetin, cyanidin chloride, shikimic acid and quinic acid; dilute HCl added until pH 2 with xanthurenic acid; dilute HCl added until pH 4 with 4-methylumbelliferone glucuronide, p-nitrocatechol sulphate, p-aminohippuric acid, succinylsulphathiazole, p-acetamidobenzoic acid and N⁴-acetylsulphanilamide; dilute HCl added until pH 5 with methyl red. The samples were then extracted with three 25 ml portions of ether; the ether extracts dried over anhydrous sodium sulphate, evaporated to dryness and dissolved in 1 ml acetone.

Chromatography

The above acetone solutions, together with appropriate standards, were examined by thin-layer chromatography on 0.5 mm thick layers of cellulose. Sigmacell Type 19 (Sigma Chemical Co.) was used with solvent 1 and MN 300 (Macherey, Nagel and Co.) was used with the other solvents. Rf values for the substrates and most of the metabolites and related compounds are listed in table 1. Chromatographic data for several phenolic compounds not shown in the table have been reported previously (SCHELINE 1966b & 1968a). Compounds were detected by their colours under ultraviolet light (350 mμ) or by the colours obtained with spray reagents. Phenolic compounds were detected by spraying with fast blue B salt followed by saturated $NaHCO_3$ and aromatic amino compounds were detected with Ehrlich's reagent.

Results

The results of the experiments in which the various test compounds were incubated with rat caecal microorganisms are shown in table 2.

The glucuronide and all the glycosides underwent extensive hydrolysis. While the aglycones were the only metabolites detected with 4-methylumbelliferone glucuronide and phenyl β-D-glucoside, the flavonoids hesperidin and rutin were found to give additional metabolites. These were mainly products resulting from ring fission and dehydroxylation although an unknown metabolite was detected in two experiments with each compound. This gave a blue purple spot with fast blue B salt at about Rf

Table 1

Thin-layer chromatography of substrates and related compounds.

Compound	Rf value × 100				
	Solvent 1	Solvent 2	Solvent 3	Solvent 4	Solvent 5
4-Methylumbelliferone glucuronide	0	75	35	51	46
4-Methylumbelliferone	36	22	93	90	69
Phenyl-β-D-glucoside	–	–	–	–	
Phenol	88	69	–	–	94
Hesperidin	0	25		57	
Ruti	0	14	66	54	
p-Nitrocatechol sulphate	0	66	–	51	46
p-Nitrocatechol	12	40	96	93	42
p-Aminohippuric acid	0	73	58	61	39
p-Aminobenzoic acid	47	52	89	87	40
Succinylsulphathiazole	1	55	84	82	40
Sulphathiazole	7	59	80	75	62
p-Acetamidobenzoic acid	18	35	91	90	57
N^4-Acetylsulphanilamide	3	66	88	76	77
Sulphanilamide	3	69	77	59	66
Methyl gallat	4	31	89	76	
O llic acid	0	25	78	55	
Pyrogallol	5	56	83	69	
Resorcinol	10	59	94	90	75
Methyl red	98	1	92	97	83
o-Aminobenzoic cid	92	59	95	96	56
N,N Dimethylphenylenediamine	11	86	74	97	94
Xanthurenic acid	0	18	48	51	14
8-Hydroxyquinaldic acid	37	32	56	78	46
(+)-Catechin	0	20		–	
Hesperetin	31	3	93	90	
Quercetin	0	0	87	–	

streaked.

See text for detection of compounds and other chromatographic data.

Solvent

1: benzene-glacial acetic acid H_2O (6 7 3 upper phase).

2 aqueous 20 / KCl-glacial acetic acid (100 1)

3 n-propanol-dioxan-0.2 M acetic acid (3:1 1)

4 n-butanol-glacial acetic acid-H_2O (4 1 5 upper phase)

5: n-propanol-0.88 mmonia (7 3)

0 42 in solvent 2. One of the rutin incubates also contained 3 4-dihydroxyphenylacetic acid.

Incubation of *p*-nitrocatechol sulphate in medium with or without

Table 2

Metabolism of compounds by the rat intestinal microflora. Test substance incubated anaerobically fo 22 hours with rat caecum extract (1 ml) in 0.1 M pH 7.4 phosphate-glucose-peptone-yeast extract medium (10 ml).

Class of compound (reaction)	Test compound	No of experi-ments	Observations
Glucuronide (hydrolysis)	4-Methylumbelliferone glucuronide	3	4-Methylumbelliferone in 3/3
Glycoside (hydrolysis)	Phenyl-β-D-glucosid	3	Phenol in 3/3
	Hesperidin	4	Hesperetin in 3/4, m-hydroxyphenylpropionic acid in 4/4
	Rutin	4	Quercetin in 4/4, m-hydroxyphenylacetic acid in 4/4 m-hydroxyphenylpropionic acid in 4/4
Ethereal sulphate (hydrolysis)	p-Nitrocatechol sulphate	3	
Glycine conjugat (hydrolysis)	p-Aminohippuric acid	6	p-Aminobenzoic acid in 6/6.
Amide (hydrolysis)	Succinylsulphathiazole	5	Sulphathiazole in 3/5.
Acetyl conjugate (hydrolysis)	p-Acetamidobenzoic acid	6	p-Aminobenzoic acid in 2/6.
	N⁴-Acetylsulphanilamide	3	No metabolites detected.
Ester (hydrolysis)	Methyl gallate	4	Gallic acid in 3/4 pyrogallol in 3/4 resorcinol in 4/4
Azo compound (reduction)	Methyl red	3	o-Aminobenzoic acid in 3/3 N,N-dimethyl p-phenylenediamine 3/3.
Phenol (dehydroxylation)	Xanthurenic acid	4	8-Hydroxyquinaldic acid in 3/4.
	Pyrogallol	4	Resorcinol in 4/4.
Heterocyclic compound (ring fission)	(+)-Catechin	5	m-Hydroxyphenylpropionic acid in 4/5
	Hesperetin	7	m-Hydroxyphenylpropionic acid in 3/7
	Quercetin	5	m-Hydroxyphenylacetic acid in 5/5 m-hydroxyphenylpropionic acid in 5/5
	Cyanidin chloride	2	No metabolites detected.
Cyclohexane derivative (aromatization)	Shikimic acid	4	Catechol in 1/4, protocatechuic acid in 1/4
	Quinic acid	6	Catechol in 1/6

See text.

caecum extract resulted in extensive hydrolysis. This could be greatly reduced or abolished by using a medium containing pH 6.6 phosphate buffer. Under these conditions, incubation for 22 hours resulted in the formation of traces of *p*-nitrocatechol in the incubates, with or without caecum extract. However another experiment showed no evidence of hydrolysis in either sample after an incubation of 18 hours. The extracts of the incubates did not contain metabolites with an aromatic amino group.

Deacetylation of *p*-acetamidobenzoic acid did not occur readily under the present experimental conditions and only one sample showed an appreciable amount of *p*-aminobenzoic acid. Another sample contained a trace of the metabolite which was not detected in any of the controls.

Fission of the heterocyclic ring occurred with three of the four test compounds used. Only the flavylium cation cyanidin chloride underwent no detectable metabolism. (+)-Catechin was converted to a small extent to *m*-hydroxyphenylpropionic acid in three samples and to a greater extent in a fourth which was incubated for 46 hours. This was the most prominent metabolite although an unknown substance giving a brown to orange colour with fast blue B salt at about Rf 0.8 in solvent 1 was detected in two incubates. An unknown metabolite found in two of the hesperetin samples showed the same chromatographic characteristics as those shown by the unknown substance obtained from hesperidin and rutin. The amounts of the *m*-hydroxyphenyl acids formed from quercetin were usually small. Two of the quercetin samples contained a metabolite which ran with the front in solvent 1 and gave a yellow colour with fast blue B salt.

Aromatization of the cyclohexanecarboxylic acid derivatives, shikimic and quinic acids, occurred in only one sample of each compound. An unknown metabolite was detected in two of the shikimic acid and four of the quinic acid incubates. This gave a yellow colour with fast blue B salt and ran with the front in solvent 1.

With the exception of *p*-nitrocatechol sulphate the test compounds were not found to undergo any change when incubated without caecum extract. Another control series was carried out using caecum extract which had been heated at 100° for 15 minutes before incubation. Using 4-methylumbelliferone glucuronide, phenyl-β-D-glucoside, methyl gallate, pyrogallol and methyl red as test substances, no metabolism was detected.

Discussion

The present methods used for investigating the metabolism of compounds by the rat intestinal microflora are identical to those used in

previous studies, where the reactions of azo reduction and decarboxylation, dehydroxylation, demethylation and reduction of double bonds in phenolic acids were demonstrated (SCHELINE & LONGBERG 1965 SCHELINE 1966a & b 1967 & 1968a). A major purpose of this investigation was therefore to determine if these techniques were also suitable for studying other types of reactions. In general the results indicate that these methods are very useful for this purpose

The finding that 4-methylumbelliferone glucuronide was readily hydrolyzed by the caecal microorganisms was expected, since it has been shown that high β-glucuronidase activity is present in the large bowel of many species (MARSH, ALEXANDER & LEVVY 1952) The importance of intestinal hydrolysis of glucuronides is manifest as many drugs, foreign compounds and their metabolites, are excreted in the bile in the form of glucuronide conjugates. Liberation of the aglycone in the intestine may establish enterohepatic circulation of the compound or it may undergo further metabolism in the gut (SMITH 1966 SMITH & WILLIAMS 1966)

The caecal microflora extensively hydrolyzed the three glycosides used in the present study Two of these compounds are flavonoid glycosides and some of the information now available on the metabolism of glycosides by the gut flora deals with these compounds. Rutin was found by BOOTH & WILLIAMS (1963b) to be metabolized to *m*-hydroxyphenylpropionic acid by rat faecal and caecal extracts. The present investigation confirmed this finding and also showed the presence of the aglycone, quercetin, in the caecal incubates. The fact that hesperidin underwent a similar reaction, together with the knowledge that it is excreted in the urine partly as the aglycone, and partly as ring fission products when administered orally to rats (BOOTH *et al* 1958) suggests that the gut flora is of considerable importance in the metabolism of this class of compound

The ability of the caecal microflora to hydrolyze ethereal sulphates has received little attention although CLOSON *et al* (1959) reported that rat intestinal bacteria hydrolyze 3,5,3'-triiodo-L-thyronine sulphuric acid ester The present results indicate a lack of activity with *p*-nitrocatechol sulphate and, in general, agree with the previous findings on the fate of arylsulphates in animals (HAWKINS & YOUNG 1954)

Relatively little is known about the metabolism of glycine conjugates by intestinal microorganisms although glycocholic acid is known to undergo splitting of the peptide bond on incubation with rat faecal suspensions (NORMAN & GRUBB 1955) The present results show that the rat intestinal flora can hydrolyze *p*-aminohippuric acid and a report by HÜLSMANN & STATIUS VAN EPS (1967) indicates that this reaction also occurs in the alimentary tract in man

The hydrolysis of other amides in the gastrointestinal tract is of practical

1-cyclohexene 1-carboxylic acid) by rats on a purified diet, resulted in the urinary excretion of catechol as well as vanillic acid (BOOTH *et al* 1960). SCHELINE (1966a) suggested that these polyhydroxy acids are converted in the intestine to 3 4-dihydroxybenzoic acid (protocatechuic acid) which can be partly decarboxylated to catechol and partly absorbed and then methylated to vanillic acid. Although aromatization was observed in only one experiment with each of the cyclohexane derivatives in the present study the detection of catechol and, particularly of protocatechuic acid in one of the shikimic acid incubates supports this suggestion.

The results obtained in the present investigation provide further evidence that the intestinal microorganisms possess numerous metabolic capabilities which canalter foreign organic compounds. Of course, studies of this type most likely reveal only some of the reactions which may actually occur in the gastrointestinal tract since many and perhaps the majority of the microorganisms growing there require special conditions for their culture This is particularly true of many strictly anaerobic types which predominate in the gut (DUBOS & SCHAEDLER 1964). Therefore, the fact that a particular reaction does not take place in experiments using simple techniques such as those used in the present work, does not necessarily mean that the reaction cannot occur in the gastrointestinal tract. Likewise, the finding that a reaction occurs in an incubate with the gastrointestinal microorganisms may greatly overestimate the significance of the reaction For example, a reaction may readily occur in the incubate due to the artificial environment which favours the growth of a microorganism which may be only a minor inhabitant of the gastrointestinal tract. Nevertheless experiments of this type are valuable not only because they can extend our limited knowledge of the metabolism of foreign compounds by the gastrointestinal flora but also because they may help to elucidate the metabolic fate of drugs and other organic compounds.

Summary

The metabolism by the rat caecal microflora of a number of drugs, foreign organic compounds or their metabolites has been studied The following classes of test compounds were used glucuronide, glycoside, ethereal sulphate, glycine conjugate, amide, acetyl conjugate, ester azo compound, phenol, heterocyclic compound and cyclohexane derivative.

The intestinal microflora brought about hydrolysis with 4-methylumbelliferone glucuronide, phenyl-β-D-glucoside, hesperidin, rutin, *p*-aminohippuric acid, succinylsulphathiazole, *p*-acetamidobenzoic acid and methyl gallate, reduction with methyl red, dehydroxylation with

xanthurenic acid and pyrogallol, ring fission with (+)-catechin, hesperetin and quercetin and aromatization with shikimic acid and quinic acid Hydrolysis of *p*-nitrocatechol sulphate and N[4]-acetylsulphanilamide by the flora was not observed No metabolites were detected in the incubates containing cyanidin chloride

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Some Pharmacological Properties of a New β-Adrenergic Blocking Agent, 1-(Isopropylamino)-3-(o-phenoxyphenoxy)-2-propanol HCl (Ph QA 33).

By

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Since the discovery of dichloroisoprenaline (DCI) by POWELL & SLATER (1958) the field of β-adrenergic blocking compounds has been the object of intensive research. As a result a large number of substances have been made, most of which may be of potential clinical interest, (for survey see BIEL & LUM 1966).

So far however only propranolol (Inderal ® ICI) has been subject to extensive clinical trials and proved effective in cardiac arrhythmias (SCHØNAU JØRGENSEN *et al* 1966), angina pectoris (GRANT *et al* 1966), and possibly in the treatment of Parkinsonian tremor (MARSDEN & OWEN 1966)

This report deals with the pharmacology of a new β-adrenergic blocking agent, Ph QA 33 using propranolol as a standard of reference (fig. 1)

Ph QA 33 was selected as the most promising member of a series of phenoxyphenoxy derivatives, prepared by K. RUBINSTEIN *et al* of this laboratory and differs from propranolol in that the aromatic part of the molecule is a phenoxyphenoxy ring whilst the latter contains a naphthoxy ring. The chemical structure of Ph QA 33 is thus closely related to 1 (isopropylamino)-3-(m-tolyloxy)-2-propanol (ICI 45 763) (CROWTHER *et*

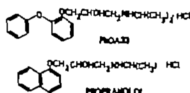


Fig. 1 The chemical structure of Ph QA 33 and propranolol.

al 1962) another potent antagonist of β-adrenergic activity (SHANKS *et al* 1966)

Preliminary experimental work with Ph QA 33 showed that it had marked β-adrenergic blocking properties As will be reported elsewhere Ph QA 33 also proved to be a potent antiarrhythmic agent with less local anaesthetic activity than propranolol. These findings justified a further study of its pharmacology

Material and Methods

Test drugs

The following compounds were used racemic 1-(isopropylamino)-3-(o-phenoxyphenoxy)- -propanol, HCl, ½ H_2O (*Ph QA 33 = QA 33*) containing 86.9% of the base Inderal ® (ICI) = propranololi hydrochloridum INN containi g 87 7% of the base, adrenalinum NFN Ilypropymalum NFN α-chloralose (BDH) DL N-Isopropylnoradrenaline hydrochloride (Fluka) = isoprenalini chloridum NFN nembutal natrium ® (Abbott) = mebumalnatrium NFN o otremorine (M y & Baker) tremorine hydrobromide, and urethanum WHO All doses mentioned in the text refer to the form of the substances given in this paragraph

A Tests for β-adrenergic blocking ffect

1 *Contraction rat Isolated guinea pig atrium* Isolated guinea pig atria were mounted in 20 ml jacketed organ bath t 28° according t the method of GREEFF *et al.* (1959). The resting tension was adjusted to 2 g. The preparatio was left for at least 30 min. or until a steady rate f atrial contractions had been reached. The frequency was determined by means of platinum wire mounted on a Starling heart lever which activated both a Thorp impulse counter (Palmer ®) d conventional telephone counter via mercury switch.

Cumulative dose-response curves for adrenaline were obtained by adding the amine to the bath in geometrically increasing doses without hanging the bath fluid. A fresh dose f drenaline was not dded until the maximal response f the previous one had been reached When the maximum response to adrenaline was obtained the bath fluid was changed repeatedly I order to allow the preparatio to recover To ensure constant sensitivity t adrenaline two dose response curves were btained n each experiment before the antagonists were tested. These were put into the bath 3 min. before the addition of the first dose of adrenaline. I this way cumulative dose-response curves were made in the presence f two different concentrations f either Ph QA 33 or propranolol. The results were expressed as a percentage of the mean maximal increase in rate of atrial contractions obtained in series of control experiments.

2. *Heart rat anaesthetized mouse* M le mice were anaesthetized with 60 mg/kg of nembutal natrium ® intraperitoneally The heart rate was determined from the ECG tracings obtained with bipolar leads according to SCHMIDEL (1933).

The mice were kept at constant body temperature of bout 37° by pplying external heat. In this way the heart rate could be maintained at stable level during the experimental period of about 30 min. Any direct influence of the ntago ists on th heart rate was established by bserving the same rat immediately before and 20 min. after the intraperitoneal dministration of the test compounds. The positive chronotropic response to isoprenaline was determined in 20 control mice by measuring the heart rate before and

one min. after an acute intravenous injection of 10 μg/kg isoprenaline. The increase in heart rate (ΔF) was then taken as a measure of the β-stimulating effect of isoprenaline. The antagonistic effect (ED_{50}) of Ph QA 33 and of propranolol was determined by giving the two substances in varying doses intraperitoneally 20 min. before the isoprenaline response was measured. All doses were tested n groups of ten mice.

3. *Blood pressure anaesthetized rat.* Rats f either sex weighing about 400 g were anaesthetized with urethane (1.5 g/kg i. p.). The trachea was cannulated with glass tube to facilitate spontaneous respiration. A polythene tube PE50 (Clay-Adams) was inserted into the right jugular vein for intravenous injections and a similar tube introduced in the left carotid artery for recording of the arterial blood pressure. The body temperature of the rats was kept at 33 by means of external he ti g. At this temperature the arterial blood pressure could be kept at a constant level for long period.

The β-blocking effect of Ph QA 33 and propranolol was assessed by their ability to prevent the vasodilator effect of isoprenaline induced stimulation of β-receptors in the peripheral vascular bed. A standard dose of 0.1 μg/kg isoprenaline was injected intra venously in a volume not exceeding 0.1 ml followed by 0.2 ml of saline. Thus the injection volume never exceeded 0.3 ml, a volume which caused only negligible rise in blood pressure. I order to obtain a constant blood pressure response isoprenaline was given t intervals f 10 min. Then QA 33 and propranolol were given intravenously in doses varying from 3–30 μg/kg, the injection volume never exceeding 0.3 ml. The degree of inhibition of the isoprenaline induced fall in blood pressure measured 5 min. later deter mined the extent of the β-blocking effect. The duration f this effect was not evaluated.

4. *Myocardial contractile force anaesthetized cat.* Eight cats f either sex weighing from 2.1–4.8 kg wer anaesthetized with allypropymal (65 mg/kg p supplemented when required during the experiment). After cannulation f the right saphenous vein for intra venous injection, and of the trachea for artificial respiration, both vagi were cut in the neck. A thoracotomy was performed the insertion being made in the 6th left intercostal space. A small Walton-Brodie strain gauge arch was sutured to the surface of the left ventricle near its base. The two sutures were so placed that the cardiac muscle was tretched to about 150% of its resting length. The myocardial contractions were recorded on an UV recorder (Ultralette ®). A dose of 1 μg/kg isoprenaline was given intravenously to obtain suitable increase in contractile force. This dose was repeated every 10 min., and when a constant response had been obtained, Ph QA 33 and propranolol were administered intravenously in doses of 10, 30 or 100 μg/kg. The reduction of the response t isoprenaline given 3 min. later was taken as an indication of β-blocking activity. In a further two cats $CaCl_2$ instead of isoprenaline was used as an unspecific cardiac stimulant. 10 mg/kg given intravenously gave reproducible increases in contractile force when repeated every 10 min. Increasing doses f om 100 μg to 3 mg/kg given intravenously of Ph QA 33 were tested and reducti of th $CaCl_2$-response 3 min. later taken as an indication of an unspecific cardiac depressant effect of QA 33

5. *Pharmacological half-life heart rat conscious rabbit.* In order to compare the duration of the β-blocking effect of Ph QA 33 and propranolol, method essentially the same as described by BLACK *et al.* (1965) was employed. Male albino rabbits weighing from 2.4–3.1 kg were trained to sit quietly in wooden bo used for pyrogen tests. Intravenous injections were made through polythene catheter inserted marginal ear vein. The heart rate was measured from the ECG according to SCHMITZEL (1933). The parameter used for the determination of the pharmacological half-lif was the isoprenaline induced increase in heart rate. In control experiments 0.1 μg/kg isoprenaline injected intravenously t constant intervals of 10 min. caused reproducible tachycardia. The pharmacological half-life was determined by recording the isoprenaline response befor and at 10 min. intervals after

the intravenous injection of 0.1 mg/kg QA 33 or propranolol. This dose did not in itself alter the heart rate significantly. The time required for 50% recovery of the isoprenaline response was regarded as a measure of the pharmacological half-life.

B. *Haemodynamics.*

1. *Arterial and venous blood pressure, ECG and respiration, anaesthetized cat.* Cats of either sex weighing from 3.3–4.8 kg were anaesthetized with chloralose (0.1 g/kg intraperitoneally). Arterial blood pressure was recorded from the right carotid artery on an UV-recorder (Ultralette ®) by means of a Statham transducer (P23AA), and the venous pressure from the left jugular vein by another Statham transducer (P23BB). By means of a Fleish tube attached to a glass cannula in the trachea and Statham differential pressure transducer (PM 197) changes in the velocity of the in- and expiratory flow were observed and recorded by the UV-writer. Volume changes were not measured. ECG was obtained by the method of SCHINKEL (1933). Heart rate was determined from the ECG. Intravenous injections were made thro gh an Olafsen cannula in the right saphenous vein. A polythene tube was inserted into the urinary bladder to avoid blood pressure changes caused by distension of this organ. The cats were kept t a constant body temperature of 38.0–38.5° by means of external heating. The substances were dissolved in saline and the doses given were 1 mg/kg intravenously (rapid injectio) 3 mg/kg intravenously (2 mg/kg/min.), and 10 mg/kg i.v. (2 mg/kg/min.). No dose was given until the effect of the preceeding one had disappeared.

2. *Resting heart rate, anaesthetized rat.* Male Wistar rats weighing from 150–200 g were anaesthetized with urethane (1.5 g/kg i. p.). The body temperature wa kept constant at bout 37° by external heating. The resting heart rate was measured every 5 minutes from the ECG. With this set up the heart rate was constant within the experimental period of one hour. After a pre-drug value had been obtained Ph QA 33 and propranolol were injected intraperitoneally in various doses, and changes in heart rat bserved for a period f 30 min. after the injection. The heart rate recorded 15 min. after the drug administration was t ken as the post-drug value at which time an effect, if any was maximal. The control group consisted of 10 rats and the test groups of five rats.

C. *Antitremorine and-oxotremorine effect mouse.*

Groups f 10 male mice of the NMRI-strain weighing from 18–25 g were used. After the initial rectal temperature had been measured by means of thermocouple galvanometer system (Elektrolaboratoriet Copenhagen) dose f either 7.5 mg/kg of tremorine or 0.1 mg/kg f oxotremorine intraperitoneally was given, and the hypothermic and analgesic effect followed by half hourly determinations. Analgesia was measured by pplying an artery clip t the oot of the tail. If the mouse did not bite after the clip within 15 sec. the test was regarded as positive. The above mentioned doses produced analgesia which was measured 30 min. after the dministration in 9 to 10 mice out of 10. This was recorded as 9/10 or 10/10 (complete analgesia) as depicted in table 5. I the same period the lowest value of the rectal temperature was also noted. The substances were given intraperitoneally dissolved in saline 30 min. bef re tremorine or oxotremorine, in volume of 0.1 ml/10 g body weight.

The effect of Ph QA 33 and propran lol by themselves n the body temperature was also investigated in order to uncover any masking effect due to an inherent hypothermic action of the test compounds in the doses used. The results of these experiments are given in the third column f table 5.

The antagonistic effect of various doses of atropine as reference substance, is also included in the table.

The results of control experiments in which saline, tremorine and oxotremorine were administered alone are given at the top of table 5, and represent the mean ± s.e.m. of 50 animals.

D *Short term toxicity*

1 *Acute oral toxicity mouse (LD50 p o.)* Male mice of the NMRI strain weighing from 18–27 g were used. The mice had free access to food and drinking water during the test. Ph QA 33 and propranolol were dissolved in sali in a volume of 0.1 ml/10 g body weight. The animals were observed for a period f one week after drug administration and the total number of deaths recorded after 24 h and 7 days. Survivors were killed by chloroform and the viscera examined macroscopically 10 mice were used at each dose level and the LD50 calculated according t the method of MILLER & TAINTER (1944) giving the mean ± s.e.m.

2. *Acute intraperitoneal toxicity mouse (LD50 p.)*.The experimental procedure was as described above with the exception that the substances were administered intraperitoneally

3. *Acute intravenous toxicity mouse (LD50 i)*. The substances were injected intravenously dissolved in saline at concentrations djusted to volume of 0.1 ml/10 g. The rate of infusion was 0.06 ml/10 sec. The LD50 was calculated as above.

4 *Acute intravenous toxicity mouse (LD100 i)*. The substances were dissolved in saline at a concentration of 0.7 mg/ml. The rate of infusion was 0.3 ml/min. giving dose of approximately 10 mg/kg/min. The infusio was continued until respiratory arrest occurred. 10 male mice were used for the determination. The LD100 was expressed as the mean ± s e.m.

5. *Acute oral t xicity cat*. Ph QA 33 and propranolol were given dissolved in saline by stomach tube to three and two cats respectively The cats were lightly anaesthetized with chloroform d ring the administration, but were awake immediately after thus allowing a proper observation of any toxic symptoms. After the acut symptoms had faded off, the cats were killed and investigated for acute pathological changes such as gastric irritation, etc.

Results

A. β-adrenergic blocking potency of Ph QA 33 and propranolol

1 *Effect on the adrenaline induced increase in contraction rate of atria in vitro* In 14 control experiments the mean contraction rate of the guinea pig atria was 107 ± 4 beats/min. After addition of geometrically increasing doses of adrenaline from 1 to 8 μg/20 ml the mean maximal rate of atrial contractions was 153 ± 6 beats/min ($\bar{x}$ ± s e.m.)

In constructing the cumulative dose response curves shown in fig. 2, the increases in contraction rate after addition of the various doses of adrenaline were calculated as a percentage of the mean maximal increase. Each point on the test curves represents the mean of four determinations.

As seen in fig. 2 incubation of the atria in concentrations of 1.5×10^{-8} and 5×10^{-9} g/ml of Ph QA 33 and propranolol respectively caused a parallel shift of the dose response curves to the right indicating a competitive antagonistic effect of QA 33 and propranolol on the chronotropic

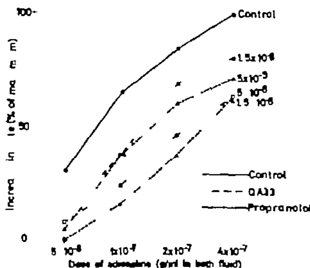


Fig. 2. Effect of Ph QA 33 and propranolol on adrenaline induced tachycardia *in vitro*. Guinea pig atria suspended in modified Tyrode solution at 28. Increases in rate of atrial contractions were produced by addition of geometrically increasing doses of adrenaline and expressed as a percentage of the mean maximal heart rate of the controls. The points necessary for construction of cumulative dose-response curves represent the mean of fourteen control experiments and of four experiments in the presence of two different concentrations of either antagonist.

action of adrenaline. Incubation in three times higher concentrations of the antagonists (5×10^{-8} and 1.5×10^{-8} respectively) caused a further shift of the dose response curve.

A quantitative comparison of the two substances shows that the lower concentrations of Ph QA 33 and propranolol increased the ED 50 for adrenaline from 7.1×10^{-8} to 1.3×10^{-7} g/ml (83 %) and 1.4×10^{-7} g/ml (97 %) while the higher concentrations caused a further increase of the ED 50 to 2.3×10^{-7} (222 %) and 2.8×10^{-7} g/ml (295 %) respectively.

The results indicate that propranolol is about three times more active than Ph QA 33 in antagonizing the adrenaline induced tachycardia *in vitro*.

2. *Effect on the isoprenaline induced tachycardia in mice.* From table 1 it is evident that doses of Ph QA 33 ranging from 0.3 to 10 mg/kg intraperitoneally did not influence the heart rate of the anaesthetized mice significantly. An exception was the 3 mg/kg dose which caused a significant increase, from 392 to 477 beats/min. This surprising observation was subsequently confirmed in a further 15 mice. At the highest dose level of 30 mg/kg the mice developed a non-significant bradycardia (470

Table I

Effect of Ph QA 33 and propranolol on isoprenaline induced tachycardia *in vivo* in mice.

Compound	Dose mg/kg i. p.	Heart rate) Before drug	Heart rate) 20 min. after drug and before isoprenaline	ΔF)	% Inhibition	ED50 mg/kg i. p.
Contr l			430 ± 12	256 ± 13		
Ph QA 33	0 3	386 ± 15	384 ± 20	203 ± 12	21	5
	1	481 ± 12	490 ± 25	141 ± 10	45	
	3	392 ± 19	477 ± 15	143 ± 18	44	
	10	454 ± 16	449 ± 21	130 ± 9	49	
	30	470 ± 32	433 ± 30	77 ± 10	70	
Propranolol	1	445 ± 22	401 ± 19	186 ± 14	27	2.6
	3	485 ± 24	423 ± 21	120 ± 16	53	
	10	462 ± 21	421 ± 20	40 ± 11	84	

) mean ± s. e. m (n = 10, except in the control group, which consisted of 20 mice)
) Increase in heart rate one min. after 10 μg/kg isoprenaline i.

to 433 beats/min.). In contrast to this, 1 as well as 3 and 10 mg/kg of propranolol caused a slight bradycardia. The difference in action of the two com pounds on the resting heart rate was also seen in the rat as described later

The mean increase in heart rate after isoprenaline injection (ΔF) was 256 ± 13 beats/min. (n = 20) Table I shows a dose dependent inhibition of the isoprenaline tachycardia after pretreatment with either test com pound A graphic plot on a semi-logarithmic scale show the ED 50's for Ph QA 33 and propranolol to be 5 and 2.6 mg/kg respectively The slopes of the two curves are, however quite different, the QA 33 curve being very flat. This makes a direct comparison of the potency uncertain. The lack of parallelism of the two curves may indicate different modes of action. A mixed β-stimulating and β-antagonistic effect of Ph QA 33 at some dose levels in contrast to the pure β-antagonist action of propranolol might explain this finding. Also in favour of this hypothesis is the different effect of the two compounds on the heart rate *per se*

3 *Effect on the isoprenaline induced blood pressure fall in the rat* Intra-

Table 2.

Effect of Ph QA 33 and propranolol on isoprenaline induced blood pressure fall in rats.

Compound	Dose μg/kg i.v.	% Reduction of initial response *)	ED 50 μg/kg i.v.
Ph QA 33	3	15 ± 4 (9)	7.5
	10	64 ± 6 (9)	
	30	100 (4)	
Propranolol	3	21 ± 7 (9)	8.0
	10	63 ± 5 (9)	
	30	88 ± 13 (4)	

*) mean ± s. e. m.
Figures in brackets indicate the number of determinations.

venous injections of doses up to 3 μg/kg of Ph QA 33 and propranolol caused no significant change in blood pressure. Doses of 10 μg/kg and upwards given intravenously consistently caused a transitory rise of about 10–15 mm Hg, an observation which has not been reproduced in the cat. A dose of 30 μg/kg of either compound almost completely abolished the blood pressure lowering effect of 0.1 μg/kg isoprenaline. In some experiments the isoprenaline response was reversed after the high doses of the β-blocking compounds. This is probably due to an unmasking of the excitatory effect of isoprenaline on α receptors as suggested by BUTTERWORTH (1963).

Table 2 summarizes the results obtained from 16 rat experiments in which doses of 3, 10, and 30 μg/kg of either test compound were given. As indicated in the table an increasing blockade of the isoprenaline response was seen ending with an almost complete block at 30 μg/kg.

Using a semilogaritmic plot the ED 50 s of both compounds are found to be approximately 8 μg/kg.

Thus, Ph QA 33 and propranolol are equipotent antagonists to the peripheral vasodilatory effect of isoprenaline.

4. *Effect on the isoprenaline and $CaCl_2$ induced increase in myocardial contractile force in the cat.* The results of these experiments are difficult to express quantitatively. Typical recordings from experiments in which 10, 30 and 100 μg/kg of Ph QA 33 and propranolol have been given

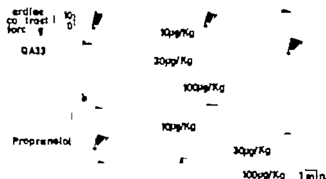


Fig. 3. Effect of Ph QA 33 (a) and propranolol (b) on the isoprenaline induced increase in contractile force. Recordings of myocardial contractions in anaesthetized cats. 1 µg/kg i. isoprenaline was given every 10 min. as indicated by the dots. The compounds were given i. v. 3 min. before isoprenaline at the signals.

are shown in fig. 3 While 10 µg/kg of either test compound given intravenously failed to influence the isoprenaline response after 3 min., 30 µg/kg as well as 100 µg/kg of Ph QA 33 almost completely abolished the positive inotropic effect of isoprenaline. In addition a clear cut negative inotropic effect of Ph QA 33 itself was observed at the highest dose level. 30 µg/kg of propranolol caused only a small reduction of the isoprenaline response while 100 µg/kg abolished it almost completely The latter dose of pro-

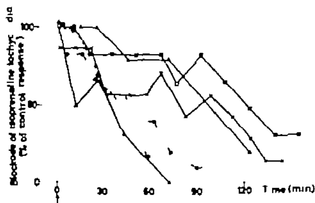


Fig. 4. Pharmacological half-life of Ph QA 33 and propranolol. Heart rate in conscious rabbits. Effect of Ph QA 33 and propranolol on the increase in heart rate produced by 0.1 µg/kg isoprenaline injected i. every 10 min. At the arrow 0.1 mg/kg of either compounds were given i. Each set of symbols represents data obtained from single rabbit. Continuous lines show results after administration of Ph QA 33 interrupted lines after propranolol.

pranolol also caused a decrease in the contractile force. These experiments indicate that Ph QA 33 is slightly more active than propranolol in its ability to block the positive inotropic effect of isoprenaline. It is also evident from the tracings that both compounds possess a myocardial depressant component.

The experiments in which $CaCl_2$ was used as an unspecific cardiac stimulant showed that increasing doses of Ph QA 33 from 100 μg to 3 mg/kg intravenously did not affect the positive inotropic effect of $CaCl_2$. The latter dose is 100 times higher than the 30 μg/kg which is able to abolish the isoprenaline response (fig. 3) This excludes the possibility that the β-adrenergic blockade produced by Ph QA 33 is due to an unspecific cardiac depression

5 *Determination of the pharmacological half-life in the conscious rabbit* The investigation was carried out in six rabbits four af which received 0 1 mg/kg Ph QA 33 intravenously and the last two the same dose of propranolol The results were plotted graphically (fig. 4) The pharmacological half-life of Ph QA 33 was 40 85 100 and 120 min respectively (average 86 min) The same figures for propranolol were 45 and 50 min. Consequently the duration of the β-blockade after Ph QA 33 is somehwat longer than after propranolol. The results obtained with propranolol agree well with those obtained by BLACK *et al* (1965) who found half lives of 40, 40, 53 and 60 min. in four rabbits.

B *Influence of Ph QA 33 and propranolol on haemodynamics*

1 *Effect on the blood pressure respiration and ECG in the cat* The effects of the intravenous administration of 1 3 and 10 mg/kg of the test compounds on the parameters indicated were tested in five and four cats respectively and the results summarized in table 3 From these data the following conclusions may be drawn

1 to 3 mg/kg Ph QA 33 caused a long lasting (>30 min.) fall in the arterial blood pressure ranging from 10 to 50 mm Hg, depending on the initial blood pressure level. If thus the level was high (high sympathetic tone) there was a considerable fall if the level was low the decrease in blood pressure was negligible The venous pressure and respiration were largely unaffected. Bradycardia was seen only in one cat after 1 mg/kg, while it was a consistent phenomenon after 3 mg/kg. The ECG changes after 1 mg/kg were insignificant while 3 mg/kg always caused a depression of the R wave and an increase of the S wave. No prolongation of the P R and R S intervals was observed.

10 mg/kg Ph QA 33 given intravenously was fatal in three of five cats. During the infusion a sustained fall in arterial blood pressure was noted.

Table 3

Effect of Ph QA 33 and propranolol on arterial and venous blood pressure, ECG and respiration in the anaesthetized cat.

Compound	Dose mg/kg i.v.	No. of expt.	Arterial blood pressure (mm Hg)	Venous blood pressure (mm H_2O)	Respiration	Heart rate (beats/min.)	ECG
Ph QA 33	1	5	acute fall and long-lasting decrease (>30') of about 10–40 mm Hg depending on initial level	increase in one cat	0	bradycardia in one cat	occasional decrease of R and increase of S wave
	3	5	acute fall and long-lasting decrease of about 20–50 mm Hg depending on initial level	increase in one cat	depression in two cats	consistent decrease of 10–90	decrease of R, increase of S wave, arrhythmia (extrasystoles) in one cat
	10	5	acute fall and long-lasting fatal decrease in three cases	increase	arrest in three cats	severe bradycardia, about 50 % decrease	serious arrhythmia, mostly irreversible
Propranolol	1	4	acute fall, long-lasting decrease (>30') of 15–50 mm Hg in four cats depending on initial level	increase in one cat	0	slight bradycardia in two cats	slight R wave depression
	3	4	acute fall and long-lasting decrease of 20–50 mm Hg depending on initial level	increase in one cat	hyperpnoea in two cats	consistent decrease of 10–70	slight and reversible R wave depression
	10	4	acute fall and long-lasting decrease of 20–80 mm Hg. In no case fatal	increase of 16–32 mm H_2O in two cats	hyperpnoea in two cats	consistent decrease of 15–75	R wave depression, S wave increase, occasional increase of PR and RS intervals. Extrasystoles

Table 4

Effect of Ph QA 33 and propranolol o the resting heart rate in rats.

Compound	Dose mg/kg i. p.	Heart rate) Before drug	15 min. after drug	/ Change
Control		397 ± 12	397 ± 14	0
Ph QA 33	1	344 ± 25	354 ± 15	+3
	3	347 ± 20	375 ± 20	+8
	10	360 ± 22	398 ± 6	+11
	30	386 ± 20	316 ± 25	−18
Propranolol	1	404 ± 7	342 ± 23	−15
	3	356 ± 10	304 ± 7	−15
	10	423 ± 17	319 ± 13	−25
	30	394 ± 27	242 ± 13	−39

) mea ± s. e. m. (n = 5, except in the control group which consisted of ten rats).

Severe ECG changes were seen at about 6 mg/kg and, since the effect on the respiration was only slight, the deaths were probably due to cardiac insufficiency caused by arrhythmia. The LD 100 was estimated to be 14 mg/kg (n = 5)

1 to 3 mg/kg propranolol given intravenously also caused a long lasting (>30 min.) decrease in arterial blood pressure (15 to 50 mm Hg), depending on the sympathetic tone. At 3 mg/kg bradycardia was a consistent phenomenon. The effect on venous pressure and respiration was only slight. The ECG changes were mild and reversible.

10 mg/kg propranolol given intravenously caused a long lasting fall in arterial blood pressure (20 to 80 mm Hg) bradycardia, and in two cats a rise in venous pressure and respiratory stimulation. The infusion was in no case fatal. The ECG changes consisted of R wave depression and S wave enhancement. Occasionally a prolongation of the P R and R S intervals and ventricular extrasystoles were observed. These changes were reversible.

Death occurred after 26 mg/kg (n = 4) and normal sinus rhythm was seen throughout the infusion. The cause of death may therefore be regarded as being due to myocardial depression.

Table 5

Effect of Ph QA 33 propranolol and atropine on tremorine a d oxotremorine induced hypothermia and analgesia.

Drug	Dose mg/kg i. p.	Saline Rectal temp. °C*)	Tremorine Rectal temp. °C	Tremorine Analgesia *)	Oxotremorine Rectal temp °C	Oxotremorine Analgesia
Saline		34.1 ± 0.1 (50)	29.0 ± 0.2 (50)	44/50	29 4 ± 0.2	46/50
Ph QA 33	1	35.5 ± 0.1	30.3 ± 0.2	6/10	29.2 ± 0.5	9/10
	3	35.2 ± 0.3	30.4 ± 0.4	4/10	30.5 ± 0.4	9/10
	10	35.2 ± 0.3	30.8 ± 0.3	0/10	31.0 ± 0.5	6/10
	30	33.9 ± 0 4	31.8 ± 0.2	0/10	30.3 ± 0.2	9/10
Propranolol	0.03	35.3 ± 0.2	29.3 ± 0.3	7/10		
	0.1	36.3 ± 0.2	30.6 ± 0.3	3/10		
	0.3	34.8 ± 0.3	31.2 ± 0.5	3/10		
	1	35 4 ± 0.2	34.3 ± 0 4	1/10	29.2 ± 0.5	9/10
	3	34.9 ± 0.3	34.0 ± 0.4	1/10	28.3 ± 0.4	10/10
	10	34.4 ± 0.2	34.2 ± 0.2	0/10	28.5 ± 0.3	10/10
	30	34 6 ± 0.3	32.8 ± 0.3	2/10	27.3 ± 0.3	10/10
Atropine	0.1	35 7 ± 0.1	30.4 ± 0.2	7/10		
	0.3	35.5 ± 0.1	30.8 ± 0.2	5/10		
	1	35.2 ± 0.2	32.4 ± 0.4	3/10	31 4 ± 0.2	7/10
	3	35.1 ± 0.2	34.0 ± 0.2	2/10	31 4 ± 0.2	9/10
	10	35.5 ± 0.2	34 7 ± 0.3	0/10	34 1 ± 0.4	0/10
	30	35.3 ± 0.1	35.4 ± 0.6	0/10	35.4 ± 0 1	0/10

) mean ± e. m. (= 10, when not indicated bracket).
) 10/10 indicates 100 analgesia, 0/10 no analgesia.

The haemodynamic studies in the cat show that Ph QA 33 and propranolol are equally potent with regard to the induction of hypotension and bradycardia in the dose range of 1 to 3 mg/kg intravenously

These experiments, however were only designed to show qualitative differences in the effect of Ph QA 33 and propranolol on the heart rate blood pressure, etc. and do not easily lend themselves to quantitative interpretations of single parameters. The increase in venous pressure was not significant until doses over 3 mg/kg had been administered. It is noteworthy that Ph QA 33 already caused severe cardiac arrhythmia

a dose of 6 mg/kg while infusion of propranolol did not change the normal sinus rhythm until lethal doses had been given (LD 100 ~ 26 mg/kg).

2. *Effect on the resting heart rate in rats* Table 4 shows that there was a marked difference between the effect of Ph QA 33 and propranolol on the resting heart rate in rats. At the low dose of 1 mg/kg propranolol given intraperitoneally caused a significant bradycardia from 404 to 342 beats/min. ($p < 0.05$), an effect which was highly significant at 10 and 30 mg/kg intraperitoneally In contrast to this, 3 and 10 mg/kg of Ph QA 33 caused a slight and insignificant increase in heart rate. A significant bradycardia (18 %, $p < 0.05$) was first observed at the high dose of 30 mg/kg of Ph QA 33

C. *Effect of Ph QA 33 and propranolol on tremorine and oxotremorine induced hypothermia and analgesia in mice*

In the upper part of table 5 the results are shown of control experiments in which saline was administered alone or followed by tremorine or oxotremorine The mean rectal temperature of 50 saline treated control mice was 36.1 $\pm$ 0.1 After administration of tremorine and oxotremorine a significant hypothermia was recorded, the rectal temperature being 29.0° $\pm$ 0.2 and 29.4 $\pm$ 0.2 respectively At the same time tremorine caused analgesia in 44 of 50 mice (44/50) and oxotremorine in 46 of 50 mice (46/50), while all the saline treated mice responded to the pain stimulus

The third column in table 5 shows the effect of Ph QA 33 propranolol and atropine on the rectal temperature when given alone. Ph QA 33 in doses of 1–10 mg/kg intraperitoneally only affected the rectal temperature slightly while 30 mg/kg caused a significant lowering. Propranolol in doses of 0.03–3 mg/kg did not affect the rectal temperature significantly Higher doses such as 10 and 30 mg/kg caused some hypothermia Atropine did not affect the body temperature in a dose range of 0.1–30 mg/kg.

Ph QA 33 had only a slight antagonistic effect on the tremorine induced hypothermia in doses of 1–30 mg/kg. In contrast, the tremorine induced analgesia was effectively antagonized, the ED 50 being 1–3 mg/kg.

Propranolol had a significant antagonistic effect on the tremorine hypothermia in doses of 1 mg/kg and upwards while the analgesic effect was already prevented at ten times lower doses. The ED 50 was 0.03–0.1 mg/kg. Atropine showed the well known effect on both parameters, the hypothermia being effectively antagonized in doses of 1–3 mg/kg and the analgesia in a dose of about 0.3 mg/kg intraperitoneally The question why all three drugs more effectively antagonize the analgesia than the hypothermia is still unresolved, but a similar phenomenon was observed when the antagonistic effect of thymoleptics on tremorine induced tremor

Table 6

Acute toxicity of Ph QA 33 and propranolol in mice.

	mg/kg)	
	Ph QA 33	Propranolol
LD50 p. o.	810 ± 86	800 ± 79
i. p.	120 ± 6	122 ± 7
– i.	36 ± 3	40 ± 1
LD100 i. v.	46 ± 3	46 ± 2

*) mean ± s. e. m. (n = 10).

and hypothermia was investigated by SPENCER (1965). Neither Ph QA 33 nor propranolol had any effect on the hypothermia and analgesia produced by oxotremorine which, however was effectively antagonized by atropine in doses of 3–10 mg/kg intraperitoneally

D *Short term toxicity of Ph QA 33 and propranolol in mice and cats*

Table 6 shows that Ph QA 33 and propranolol are equally toxic in mice regardless of the route of administration. The figures also indicate that both substances are readily absorbed from the gastro-intestinal tract as the ratio LD 50 orally/LD 50 intraperitoneally is only approximately 7 All deaths occurred within 24 h so the LD 50 values for 24 h and those after one week are identical. Convulsions were seen at toxic dose levels. At autopsy of the surviving animals one week after the administration nothing abnormal was observed.

In the cat experiments two dose levels of either substances were tested

25 mg/kg of Ph QA 33 orally caused only occasional vomiting, anorexia and slight mydriasis, while

50 mg/kg of Ph QA 33 orally caused occasional vomiting, anorexia, a sick appearance and severe hypernoea. This dose was therefore considered as being definitely toxic.

Propranolol given in the same doses caused almost the same symptoms. It therefore appears that in the cat too the two substances are equally toxic.

Discussion

The classification of Ph QA 33 as a β-adrenergic blocking agent is evidenced by its antagonistic effect on adrenaline or isoprenaline induced changes, using one *In vitro* and four *in vivo* techniques. The nature of

the β-adrenergic blockade is clearly demonstrated on the isolated guinea pig atria where increasing concentrations of Ph QA 33 caused a parallel shift of the cumulative adrenaline dose-response curve to the right. This indicates that the antagonism is of a competitive type. The dose-response curves for Ph QA 33 were parallel to those obtained with propranolol which has previously been shown to be a specific β-receptor antagonist (BLACK *et al* 1965) The specificity of Ph QA 33 is evidenced by the finding that a dose 100 times larger than that necessary to abolish the isoprenaline induced increase in myocardial contraction *in vivo* did not affect the $CaCl_2$-induced contraction. The activity of Ph QA 33 as compared to propranolol varies with the experimental model, but is of the same order of magnitude. A comparison of the propranolol data reported here with those obtained by other investigators is difficult because of differences in experimental design, but the results obtained on the isolated atria agree well with those reported by BLACK *et al* (1965) who used a slightly different technique. The duration of the β-blockade assessed from the rabbit *in vivo* experiment shows that the pharmacological half life of Ph QA 33 is slightly longer than that of propranolol. The results obtained with the latter compound are also in agreement with the data of BLACK *et al* (1965)

The results obtained on the isoprenaline induced tachycardia in mice suggest that a β-adrenergic stimulant component might be involved in the action of Ph QA 33 while propranolol belongs to the group of pure β-antagonistic compounds. In addition the finding that the resting heart rate in mice was either unaffected or increased by Ph QA 33 but lowered after propranolol is in favour of some intrinsic sympathomimetic activity of Ph QA 33 This kind of "intrinsic activity" (ARIENS & SIMONIS 1961) has been reported to be present in several groups of antagonists and has also been found in other β-blocking compounds (ÅBLAD *et al.* 1967). These authors suggest that β-adrenergic blocking agents with a stimulant component possess therapeutic advantages over the pure antagonists because they would be expected to be devoid of cardiac depressant properties within a reasonable dose range, and would thus leave the resting heart rate and thereby cardiac output unaffected. As a representative of the pure β-blockers, propranolol has a consistent lowering effect on the cardiac output due to elimination of the endogenous sympathetic tone on the heart. If the sympathetic tone is high this depressant effect may be dramatic and even fatal if left untreated (ROSÉN 1966) while the cardiac depression of β-blocking agents is negligible under conditions in which the sympathetic tone is low (PIPPIG 1965).

Evidence of a possible β-adrenergic stimulant effect of Ph QA 33 was further obtained by the experiments on urethane anaesthetized rats in

which 1 mg/kg of propranolol given intravenously already caused some decrease in the resting heart rate while a thirty times larger dose of Ph QA 33 was necessary to cause a significant bradycardia. This finding may indicate a smaller effect of Ph QA 33 on the cardiac output than the one observed after propranolol and as such may prove beneficial clinically

The results of the haemodynamic studies in the cat confirm the observation that cardiac depression by β-blockade as estimated by a decrease in blood pressure, depends largely on the level of the sympathetic tone. If the initial blood pressure was high both Ph QA 33 and propranolol had a marked hypotensive effect, while this action was negligible if the initial blood pressure was low The studies in the cat also revealed a significant difference between Ph QA 33 and propranolol on the ECG Propranolol did not affect the normal sinus rhythm until very high doses were infused (>20 mg/kg) while Ph QA 33 at a dose level of 5 mg/kg intravenously already caused severe irregularities. This difference has probably no clinical relevance as the β-blockade takes place using doses which are only about 1 / of the dose at which cardiotoxicity appears. Nevertheless, the finding that Ph QA 33 caused cardiac arrhythmias was surprising, particularly since this substance was found to be a potent antagonist of ouabain induced arrhythmias in guinea pigs

Beta-adrenergic blocking compounds have been reported to be effective in the treatment of Parkinson's disease (MARSDEN & OWEN 1966), the rationale for this being that emotional stress and administration of adrenaline increase Parkinsonian tremor Antagonism of tremorine and oxotremorine induced hypothermia and analgesia is a commonly used method for studying compounds with a possible effect on extrapyramidal tremor (CHEN 1958 SPENCER 1965). JACOBI (1967) found propranolol as well as two other β-blockers to be effective against tremorine induced tremor in mice.

In our technique in which atropine used as a well established anti parkinsonian agent was fully effective against tremorine as well as oxotremorine induced hypothermia and analgesia, Ph QA 33 and propranolol were only active against changes induced by tremorine, but not by oxotremorine (table 5). This observation indicates that Ph QA 33 and propranolol act only by interfering with the hepatic biotransformation of tremorine to the active metabolite oxotremorine, an explanation which would account for lack of effect against both agonists of certain thymoleptics (SJÖQUIST & GILETTE 1965). Ph QA 33 and propranolol should then be classified together with thymoleptics and phenothiazines in the group of tremorine antagonists ineffective in the treatment of Parkinson's disease (LESLIE & MAXWELL 1964). Clinical results of propranolol ha

also been contradictory and doubleblind cross-over trials have given disappointing results (VAS 1966)

A new trend in the field of β-blocking agents is the development of substances with selective β-blocking properties. The aim is to discover compounds which will block only one or a few receptor areas, leaving the others intact. To be successful in these efforts the assumption that not all β-receptors are equally sensitive to blockade, i.e. that the nature of the receptors differs from one tissue to another must be valid Experimental work with DCI and the corresponding α methyl derivative by MORAN (1966) has made this probable. He found that while DCI antagonized the cardiac stimulant and vasodilator effect of isoprenaline equally well α methyl DCI clearly produced a selective antagonism of the vasodilatory effect, but not of the positive inotropic effect of isoprenaline. Recently BARRETT *et al* (1967) have described another compound, 4-(2 hydroxy 3-isopropylaminopropoxy)acetanilide (ICI 50,172) which blocked the positive chronotropic and inotropic effects of isoprenaline in doses less than 1 / of that necessary to block the peripheral vasodilatory effect.

A consequence of this is the theoretical possibility of blocking adrenergic receptors in the sino-atrial node and the conducting tissue of the heart, without affecting the β-receptors in the ventricular myocardium. The decreased myocardial adaptability present during β-adrenergic blocking therapy and representing a serious drawback in the use of these compounds as antiarrhythmics, could then be eliminated Propranolol has been shown to block the isoprenaline induced changes in cardiac contractile force, heart rate, and peripheral vasodilatation equally well and must therefore be regarded as a non-selective β-blocking compound (SHANKS 1966)

With regard to a possible selective effect of Ph QA 33 the experimental work reported in this paper does not allow definite conclusions. The studies on the antagonism of the isoprenaline induced changes in myocardial force, heart rate and peripheral vasodilation have been performed in different species and against different doses of agonist. Valid potency ratios are therefore not available However a few cat experiments in which Ph QA 33 was tested against the vasodilator effect of isoprenaline showed that about 10 μg/kg intravenously was necessary to block the peripheral action of this amine. This corresponds quite well to the dose required to block the positive inotropic effect in the same species (fig. 3). It is therefore unlikely that Ph QA 33 has a selective blocking effect.

Summary

1. The β-adrenergic blocking activity of Ph QA 33 has been evaluated in one *in vitro* and four *in vivo* techniques and found to be equal to that of propranolol. The specific β-blocking effect of Ph QA 33 is combined with weak β-adrenergic stimulant properties, while propranolol was found to be a pure β-receptor antagonist.

2. Haemodynamic studies with the two compounds show similar features. In contrast to propranolol Ph QA 33 caused cardiac arrhythmias in high doses. This was, however, not regarded as prohibitive for its possible use as a β-adrenergic blocking agent.

3. Propranolol was highly effective and Ph QA 33 partly effective in antagonizing hypothermia and analgesia produced by tremorine, while they were both ineffective against similar changes caused by oxotremorine.

4. Short term toxicity in mice and cats showed that the acute toxicity of the two compounds is very similar.

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The use of ³H DOPA for Studying Cerebral Catecholamine Metabolism

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Isotopically labelled amines have been of great value in the investigation of peripheral sympathetic transmitter mechanisms. The corresponding central mechanisms are more difficult to investigate since noradrenaline and many of its analogues do not readily cross the blood brain barrier (WEIL MALHERBE *et al* 1961) Some investigators have tried to overcome the blood-brain barrier by introducing the amines intraventricularly or intracisternally (MILHAUD & GLOWINSKI 1962 & 1963 GLOWINSKI KOPIN & AXELROD 1965 SCHANBERG SCHILDKRAUT & KOPIN 1967) The distribution of amines administered by this route however differs considerably from the physiological pattern (FUXE & UNGERSTEDT 1966) Another possibility is to administer labelled precursors (tyrosine or 3,4-dihydroxyphenylalanine, DOPA) which readily pass the blood-brain barrier and are then transformed into catecholamines. One advantage of this method is the possibility of studying and comparing central and peripheral catecholamine metabolism in the same experiments. The use of labelled tyrosine provides possibilities for estimating the rate of formation and turnover of the catecholamines under various conditions (UDENFRIEND & ZALTZMAN-NIRENBERG 1963) There are, however certain complications involved in the use of labelled tyrosine. The yield of labelled catecholamines is relatively low (BURACK & DRASKÓCZY 1964), and tyrosine is involved in several other metabolic pathways. Furthermore, labelled tyrosine appears to be available for the catecholamine synthesis for at least 10–12 hours after its administration (UDENFRIEND & ZALTZMAN-NIRENBERG 1963), which makes the distinction between catecholamine synthesis and metabolism difficult. In con-

Table 1

Elution procedure for DOWEX 50W X4 cation-exchange column.

Sample	Eluant	ml	Fraction
Effluent from the alumina column	2N HCl	8	discarded
		9–20	normetanephrine
		21–24	intermediate fraction
		25–44	methoxytyramine
Eluate from the alumina column	N HCl	9	discarded
		10–20	noradrenaline
		21–27	intermediate fraction
		28–43	dopamine

trast, DOPA enters the catecholamine synthesis below the rate limiting step (the hydroxylation of tyrosine) and is rapidly converted to catecholamines, and the yield of labelled amines is higher than after tyrosine.

Specifically labelled L-^{3}H DOPA(ring 2,5 6-^{3}H) with a very high specific activity (35 curies/mM) has recently become available. With this new tool we have tried to label the endogenous stores of catecholamines using a dose of ^{3}H DOPA (5 µg/kg) which is not expected to interfere with the normal catecholamine metabolism.

Material and Methods

^{3}H L DOPA, 5 µg/kg, was given intravenously to mice. The animals were divided at random into groups of six. After various time intervals the animals were decapitated and the brains and hearts removed and extracted in perchloric acid (PCA) as described by Bertler, Carlsson & Rosengren (1958). Twenty micrograms of unlabelled noradrenaline (NA), dopamine (DA), normetanephrine (NM) and methoxytyramine (MT) were added to each sample before the homogenisation. After addition of 2 mg ascorbic acid, 20 mg EDTA, 1 ml of 2 N sodium acetate and subsequent adjustment of the pH to 6.5 by means of 5 N potassium carbonate, the extract was put on an alumina column essentially as described by Rutledge & Weiner (1967). The effluent from the column was put as soon as possible (within 30 minutes) on Dowex 50 W x 4 cation-exchange column (diameter 4.0 mm height t pH 1 0 128 mm buffered with 25 ml of an 0.1 M phosphate buffer containing 10.74 g Na_2HPO_4 $12H_2O$ and 9.52 g KH_2PO per litre) or if this could not be done, it was acidified with 1 ml 4 N PCA. In the latter case another 2 mg of ascorbic acid was added and the pH again adjusted to 6.5 with potassium carbonate before the ion-exchange step. The alumina column was eluted with 15 ml 0.4 N PCA. The eluate from the alumina step was also adjusted to pH 6.5 with potassium carbonate after the addition of 2 mg ascorbic acid and 20 mg EDTA and then put on another DOWEX 50 column. When the ion-exchange

step was not carried out on the same day as the alumina step the samples were stored at bout −20° In no case was the ion-exchange step performed later than 4 days fter the tissue extraction.

After washing with 40 ml of water the DOWEX columns were eluted as described in table 1 The intermediate fraction served as a control of the separation. The radioactivity of the eluates was measured by liquid scintillatio counting as was described elsewhere (WALDECK 1968)

When ^{3}H DA or ^{3}H NA were added to non-radioactive tissue extracts and carried through the whole analytical procedure, about per cent of the total recovered activity were found in the MT and NM fractions.

I some experiments the recovery of the carrier amines was estimated by measuring the native fluorescence. The mean ± S D of 17 (NA nd DA) and 35 (NM and MT) such determinations was as follows 78 % ± 10 (NA), 78 % ± 7 (DA), 81 % ± 10 (NM) and 79 % ± 7 (MT). No corrections were made for recovery L 3(3 4-dihydroxyphenyl)alanine (ring-2,5,6-^{3}H) (^{3}H-DOPA) with specific activity of 35 curies/mM was btained from The Radiochemical Centre, Amersham, England.

Results

The specificity of the analytical procedure was tested in the following experiment. Nialamide, 50 mg/kg i.p. was given to mice 2 hours before giving 5 µg/kg ^{3}H DOPA intravenously Sixty minutes after the administration of ^{3}H DOPA the animals were killed and their brains removed and extracted in PCA. The extracts were carried through the whole analytical procedure as described under methods except that elution of the DOWEX columns was performed in 1 ml fractions. The fluorescence and radioactivity of each fraction was measured. The results are presented in fig. 1 The eluate of the alumina column when subsequently placed on a DOWEX column and eluted, yielded two peaks of radioactivity These two peaks corresponded to the elution pattern of the carrier NA and DA, respectively The alumina effluent yielded two peaks of radioactivity corresponding to the fluorescence peaks of carrier NM and MT

Animals were killed at various intervals after intravenous administration of ^{3}H DOPA. ^{3}H DA, ^{3}H NA, ^{3}H MT and ^{3}H NM in the brain and the heart were determined ^{3}H DA was rapidly formed from ^{3}H DOPA in the brain (fig. 2) The time-course of this amine seemed to be diphasic with one maximum at 7 5 min. (0.13 pmol/g)*) and another at about 2 hr During the first four hours after the ^{3}H DOPA injection ^{3}H NA in the brain increased to 0.22 pmol/g, which is 4 times more than the simultaneously observed ^{3}H DA. From that time ^{3}H NA started to decrease, and 16 hr after the injection of ^{3}H DOPA 0.02 pmol ^{3}H NA remained The level of ^{3}H-MT at 7 5 min. was about the same as that

*) Corresponding to 21 pg/g 1 pg (picogram) = 10^{-12} gram).

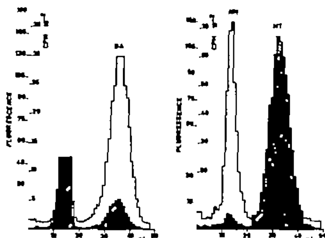


Fig. 1 Ion-exchange chromatography of basic metabolites formed from ^3H DOPA in the mouse brain. The tissue extract was first passed through an alumina column. The eluate (left) and the effluent (right) from this column were then put on separate DOWEX 50 columns. Shaded area radioactivity Unshaded area fluorescence of carrier noradrenaline (NA), dopamine (DA), normetanephrine (NM) and methoxytyramine (MT) added to the extract. For further explanation see text.

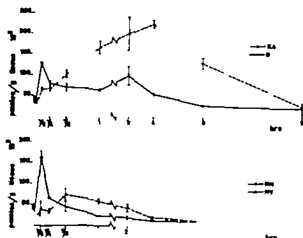


Fig. 2. Time-course of ^3H-noradrenaline (NA), ^3H-dopamine (DA), ^3H-normetanephrine (NM) and ^3H-methoxytyramine (MT) in the mouse brain after the i. v. administration of 5 μg/kg ^3H DOPA. The mean ± s. e. m. of 3 (6 at 2 hr) experimental groups, each group consisting of 6 animals are shown.

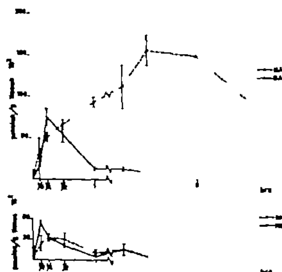


Fig. 3. Time-course of ^{3}H-noradrenaline (NA), ^{3}H-dopamine (DA), ^{3}H-normetanephrine (NM) and ^{3}H methoxytyramine (MT) in the mouse heart after the i. administration f 5 µg/kg ^{3}H DOPA. The mean ± s. e. m. f 3 (6 at 2 hr) experimental groups, each group consisting of 6 animals are shown.

of its parent substance ^{3}H DA but it then decreased rapidly The time course of ^{3}H NM differed from that of ^{3}H MT in that the maximum was reached about 20 min. later After 8 hr both the O-methylated amines had reached very low levels.

In the heart ^{3}H DA reached an early maximum (fig. 3), but disappeared more rapidly than in the brain. Eight hours after the administration of ^{3}H DOPA, the ^{3}H DA was very low ^{3}H NA increased during the first 4 hr to 1.5 pmol/g. About the same level was found 4 hr later after which the ^{3}H NA started to disappear Sixteen hours after the injection of the labelled DOPA 0.46 pmol ^{3}H NA/g were left. The 3-O-methyl derivatives 3H MT and ^{3}H NM showed about the same time course in the heart as in the brain. It should be noted, however that the amount of these amines in the heart was lower in relation to their parent compounds than in the brain although the absolute concentrations were higher

Discussion

In the present investigation a combination of alumina and cation-exchange columns was used in order to separate all the four basic metabolites of DOPA *i.e.* DA NA, MT and NM As shown in fig. 1 a complete

separation on one single DOWEX 50 column of the size used would be impossible since NM and DA would mix. A column long enough to separate these two amines was tested but was found to be too cumbersome

There seems to be general agreement that the major part of the brain DA and NA is stored in separate neurons, here called the DA and NA neurons, respectively However after the administration of labelled DOPA, the possibility should be considered that the amines are formed in sites where they normally do not occur or occur in very low concentrations. The rather complicated course of the ^{3}H DA and ^{3}H NA curves obtained in the present investigation indicates that different catecholamine pools exist in the brain but it is impossible to tell from the present data where these pools are located. For example, the ^{3}H DA curve of the brain seemed to take a diphasic course, with one maximum at 1.5 min and another at about 2 hr It is reasonable to assume that the slower phase represents partly at least the DA turnover of the bulk of the brain DA, which is located in the specific DA-storing neurons. The interpretation of the early ^{3}H DA peak is more difficult. Several possibilities may be considered *i.e* this peak represents 1) the ^{3}H DA in the blood 2) the ^{3}H DA formed in the capillary walls (BERTLER *et al* 1963 & 1966 FUXE & OWMAN 1965 OWMAN & ROSENGREN 1967) 3) intraneuronal pools with rapid turnover in DA, NA or 5-hydroxytryptamine (5-HT) neurons. It is interesting to note that the early ^{3}H DA peak coincides with the ^{3}H MT peak. Since catechol-0-methyl transferase (COMT) appears to act chiefly on extraneuronal catecholamines (CARLSSON & HILLARP 1962 KOPIN & GORDON 1962), this suggests either that the ^{3}H DA is formed extraneuronally or that it is released into the extraneuronal space soon after formation.

The brain ^{3}H NA curve had a somewhat less complicated shape. NA synthesis probably does not occur to any considerable extent outside the NA neurons, and therefore the ^{3}H NA curve probably represents processes taking place in or at these neurons.

It is interesting to note that the ^{3}H NA increased continously during the first 4 hr of the experiment. Since DA is the immediate precursor of NA one might expect that the formation of ^{3}H NA occurred at the expense of an equimolar amount of ^{3}H DA As shown in fig. 1 this was not the case. No explanation of this phenomenon can be offered at present. It may be that ^{3}H DOPA is available for ^{3}H NA synthesis for at least 4 hr After administration to rats of labelled DOPA in a dose of 20 mg/kg labelled amino acids were found in the blood and brain for several hours (GEY & PLETSCHER 1964) but it is not known to what extent the radioactivity represented DOPA itself or its 3-0-methylated

metabolite, which has been found in the brain in large amounts after the injection of ^{3}H DOPA (PERSSON & WALDECK, to be published).

The ^{3}H NM had a maximal concentration about ½ hour after the pre cursor injection and then declines slowly for about four hours to very low levels. This amine reached its maximum long before its parent amine (^{3}H NA) This suggests that ^{3}H NA moves from a pool, from which it is easily released, e.g by nerve impulses, to a more stable pool

One hour after the injection the ^{3}H NA levels were much higher than the ^{3}H DA levels. This is remarkable, since in the brain DA is present in higher amounts than NA (BERTLER & ROSENGREN 1959). However a similar ratio between DA and NA formed from labelled DOPA is reported by BURACK & DRASKÓCZY (1964) and SPECTOR *et al* (1965) When labelled tyrosine is used, more DA than NA is formed (UDENFRIEND & ZALTZMAN-NIRENBERG 1963 SPECTOR *et al* 1965 UDENFRIEND *et al* 1966 GORDON *et al* 1966). A possible explanation for this difference may be a different distribution of labelled DOPA and labelled tyrosine. However some *in vitro* experiments (YOSHIDA *et al* 1963) and some *in vivo* experiments (c.f GEY & PLETSCHER 1964) indicate that the same uptake mechanism for DOPA and tyrosine operates. Another explanation is, that tyrosine hydroxylation is more rapid in dopamine neurones than in noradrenaline neurones, while the inverse may be true of DOPA decarboxylation. With increasing doses of labelled DOPA (1 mg/kg or more) the formation of labelled DA increases more than that of NA and the ratio is inverse (UDENFRIEND & ZALTZMAN-NIRENBERG 1963 UDENFRIEND *et al* 1966 BURACK & DRASKÓCZY 1964 RUTSCHMANN *et al* 1965). While the tyrosine hydroxylation step is thought to be the rate limiting step in the overall catecholamine synthesis, DOPA decarboxylation has a large and dopa mine-β-hydroxylation a moderate capacity (c.f HESS *et al* 1961). The greater rise in DA than NA after large doses of DOPA suggests that under these conditions the last mentioned step is rate-limiting.

In heart the ^{3}H NA curve was similar to that of the brain although the data indicated a slower turnover This is in agreement with earlier observations (BURACK & DRASKÓCZY 1964) The ^{3}H DA, which in the heart, in contrast to the brain should serve essentially as a precursor of ^{3}H NA, had an early single peak at ½ hr The rise in ^{3}H NA between 1 and 4 hr did not occur at the expense of a corresponding amount of ^{3}H DA in the heart. The interpretation of this phenomenon is difficult, since both ^{3}H DA and ^{3}H NA may reach the heart via the blood from other parts of the body (c.f the above discussion of the corresponding brain data). For similar reasons the early ^{3}H MT and ^{3}H-NM peaks in the heart are difficult to interpret.

In brain the ^{3}H NA concentration was only ⅓th of that in the heart,

whereas the endogenous NA levels in these tissues are more similar This difference may be due to the blood brain barrier As mentioned above, the transport of ^{3}H DOPA across the blood brain barrier is accompanied by a loss through decarboxylation in the capillary walls, and hence the precursor concentration is lower in brain noradrenaline neurones than in those of the heart (c.f GEY & PLETSCHER 1964)

Summary

^{3}H DOPA of high specific activity was given intravenously in small doses (5 μg/kg) to mice. At various time intervals the animals were killed and extracts made of the brain and heart. By means of alumina and Dowex 50 columns the labelled dopamine (^{3}H DA) noradrenaline (^{3}H NA) methoxytyramine (^{3}H MT) and normetanephrine (^{3}H NM) were separated and determined.

The time curves for ^{3}H DA and ^{3}H NA were considerably lower in the brain than in the heart, probably because of limited penetration of ^{3}H DOPA through the blood-brain barrier

The ^{3}H DA curves of the two tissues had different shapes, with only one peak in the heart and a complex, apparently biphasic course in the brain. In part this difference reflects the different functions of dopamine in the two tissues – in the heart a simple precursor function, while in the brain it also has an independant role.

The ^{3}H NA curves of the two tissues were similar though there was an indication of a more rapid turnover in the brain. The accumulation of ^{3}H NA continued for several hours did not take place at the expense of a corresponding amount of ^{3}H DA.

The ^{3}H MT and ^{3}H NM curves showed single peaks. The course of the ^{3}H NM curve in the brain suggested the existence of a small nor adrenaline pool, which is rapidly released into the extraneuronal space, possibly by the nerve impulse

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Antigenic Histamine Release from Fractionated and Unfractionated Peritoneal Cells from Sensitized Rats

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Histamine is released from isolated peritoneal mast cells from sensitized rats on incubation *in vitro* with specific antigen (UVNÄS & THON 1959 PERERA & MONGAR 1963 JOHNSON & MORAN 1966). This also occurs when the mast cells have not been isolated from other peritoneal cells (GARCIA AROCHA 1961 NORN 1965) The histamine release is caused by a specific antigen-antibody reaction (NORN 1967b)

The present study was done in order to determine whether the histamine release in a peritoneal cell suspension of different cells (*PCS* that is unfractionated cells) is derived exclusively from the mast cells, uninfluenced by the other cells and substances contained in the suspension It was also investigated whether the histamine release was dependent on the mast-cell count, on the antigen concentration and on the incubation period. Further it was investigated whether the histamine release was influenced by a fraction procedure consisting of a differential centrifugation by bovine serum albumin to obtain a suspension with partially isolated mast cells (*FPCS*).

Experiments and Methods

Horse serum

Inactivated horse serum, heated at 56 for half an hour

Non-sensitized rats

Female albino rats, weighing 160–200 g.

Sensitized rats

Female albino rats weighing 120–150 g, sensitized t horse serum (NORN 1965) or egg albumin (NOR 1967b).

Modified Tyrode solution

This is a buffer solution (pH 7.1) containing 1.44 × 10^{-1} M NaCl, 3 × 10^{-3} M KCl, 8 × 10^{-4} M $CaCl_2$, 8 × 10^{-3} M Na_2HPO_4, and 3 × 10^{-3} M KH_2PO_4.

Peritoneal cell suspension (PCS)

A suspension of peritoneal cells removed from each rat as described by NORN (1967b).

Duration of histamine release

PCS from rats sensitized to horse serum were pooled. From this pool samples of 3.50 ml were taken, heated to 37° and incubated with 250 µl horse serum for 30 sec., 2, 10, and 30 min. respectively. Corresponding samples cooled to 6° were mixed with horse serum at the same temperature, but not incubated at 37° (blank samples). Immediately afterwards all samples were centrifuged at 1500 × g for 15 min. at 6°. Thereafter the quantity of released histamine was determined as the per cent of total histamine content (NORN 1967a), but with no correction for the small amount of histamine released by the mechanical manipulation. The results are shown in table 1.

Role of mast-cell count

Similar samples were taken from the pooled PCS and from dilutions of the pool with modified Tyrode solution in the ratio 1 + 3 and 1 + 9. After incubation at 37° for 30 min. with 250 µl horse serum, the samples were centrifuged at 1500 × g for 20 min. at room temperature, and the quantity f histamine released determined as th percentage of the total content in the sample (table 2).

Role of horse-serum concentration

F m the pooled PCS, samples of 2.75 ml were removed and incubated for 30 min. at 37° with 1.00 ml horse serum or with the same lume of horse serum in various dilutions with modified Tyrode solution. Th histamine release is illustrated in fig. 1.

Fractionation of peritoneal cell suspensions by differential centrifugation

To obtain a cell suspension containing more mast cells in relation to leucocytes, the PCS was fractionated by differential centrifugation as follows. Into siliconized, conical centrifuge tube, 1.0 ml 35 % (w/v) bovine serum albumin was placed, and above this 8 ml PCS was placed cautiously taking care that the two fluid phases did not mix. Centrifugation was performed t 160 × g for 8 min. Thereafter the upper phase and the layer of cells between the phases were removed. The sediment, which was high in mast cell content, was carefully suspended in the albumin layer and the suspensio transferred to a centrifuge tube in which it was mixed with 9 ml f modified Tyrode solution. The mixture was then centrifuged at 160 × g for 8 min. The supernatant fluid was removed and the cellular sediment carefully suspended in modified Tyrode solution. The suspension is called *fractionated PCS (FPCS)*.

Cell content of fractionated and unfractionated PCS

The mast cell and leucocyte content was counted in counting chamber after staining with toluidine blue (LAGUNOFF & BENDITT 1959). The percental distribution of leucocytes was determined by differential counting in smears stained by the method of May-Grünwald.

Correlation between histamine and mast-cell content in fractionated and unfractionated PCS

From each rat sensitized to horse serum, a PCS sample was removed, coagulation being prevented by the addition of heparin (2.5 i.u/ml suspension). To btain sufficient quantities of PCS, the samples from 2 rats were pooled. An aliquot was removed for fractionation. In the samples of the fractionated PCS (FPCS) as well as in the unfractionated PCS, the number of mast cells and leucocytes, as well as the total histamine content were determined (table 3) – as well as the distribution of leucocytes (table 4) and the release of histamine following incubation with horse serum (final concentration 6 %) at 37° for 30 min. (table 5).

Role of fractionation medium

From each of 4 rats sensitized to horse serum, 2 samples of PCS, 6.0 ml, was removed, centrif ged at 120 × g for 10 min., and the supernatant removed. One sediment was suspended in 1.0 ml 35 % (w/v) bovine serum albumin and the other in 1.0 ml modified Tyrode solution while being gently shaken. The samples were left to stand for 20 min., at room temperature. After addition of 9 ml modified Tyrode solution to all samples, the samples were cautiously turned upside down and centrifuged at 120 × g for 10 min. The supernatant was removed and the cell sediments suspended in 3.5 ml modified Tyrode solution. 250 μl horse serum was added, and the samples were incubated for 30 min. at 37° The histamine release is listed in table 5.

Possible liberation of mast-cell disrupting factor

T investigate whether there might be, during the incubation of PCS from horse serum sensitized rats with horse serum, a liberation of a mast-cell disrupting facto which then releases histamine from the mast cells, the following experiments were performed 35 ml of the named PCS was incubated for 30 min. at 37° with 2.5 ml horse serum. After centrifugation the cell-free supernatant was removed and investigated for possible disrupting f ctor by the ability of the supernatant to release histamine, partly in PCS from non-sensitized rats and partly in similar suspension from rats sensitized to egg albumin.

The investigations were carried out as follows 3.75 ml supernatant was added to the cell sediments from 3.5 ml PCS from non-sensitized rats and from rats sensitized to egg albumin. The cells were carefully suspended, and the samples were incubated at 37° for 30 min. Thereafter the quantity of released histamine was determined as the per cent of the total histamine content of the sample, correcting for the amount of histamine added with the supernatant (table 6). T compare this histamine release with the release which occurs during the mechanical manipulation of the cells, the cell sediments from corresponding PCS were incubated with modified Tyrode solution, instead of the named supernatant.

Results

Duration of histamine release (table 1)

During incubation of PCS with horse serum an equally large percentage of the total histamine content of the sample was released in the course of 30 seconds as in the course of the longer-lasting incubation (2, 10, or 30 min). In all cases about half the histamine content was released. With no incubation, only about 5 % of the total histamine content was released.

Table 1

Duration of histamine release in suspensions of peritoneal cells from rats sensitized to horse serum on incubation with horse serum. The figures indicate the release of histamine in per cent of the total content. Three determinations are given f r each sample.

	Histamine release in per cent				
	Incubation period (minutes)				
Control	0.5	2	10	30
4	54	54	53	54
5	54	54	51	53
4	51	53	50	50

Role of mast-cell count

Table 2 shows that in the three samples with different mast-cell counts per ml sample, a constant percentage of the total histamine content (approx. 50 %) was released on incubation with horse serum.

Role of horse-serum concentration

From fig 1 it can be seen that the histamine release in PCS appears to increase in proportion to the logarithm of the horse-serum concentration.

Table 2

Release of histamine by horse serum in samples taken from suspension of peritoneal cells from rats sensitized to horse serum. The samples were used undiluted or diluted (1 + 3 or 1 + 9) with modified Tyrode solution. The release of histamine is expressed in per cent f th total content of the sample. Three determinations for each sample.

Dilution f suspension of peritoneal cells	Histamine release, in per cent, by horse serum		
undiluted	57	54	50
1 + 3	56	52	50
1 + 9	59	52	48

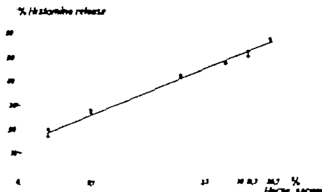


Fig. 1 Release of histamine brought about by various concentrations of horse serum in suspensions of peritoneal cells from rats sensitized to horse serum. Ordinate: Release of histamine in per cent of the total content of samples taken from pool of peritoneal cell suspensions. Abscissa Concentration of horse serum in the sample (per cent) Each point represents single determination.

Cell content in fractionated and unfractionated PCS

As seen in tables 3 and 4 the *unfractionated PCS* contained mast cells (about 3 %), lymphocytes (75 %), eosinophilic leucocytes (18 %) poly morphonuclear neutrophils (3 %), and monocytes (2 %). The *fractionated PCS* contained a higher percentage of mast cells (6–60 %) and a correspondingly lower percentage of leucocytes. Differential counting of the leucocytes revealed the same distribution in fractionated and unfractionated PCS (table 4) Apart from the above mentioned cells, the suspensions contained a varying number of mesothelial cells and erythrocytes

Correlation between histamine and mast-cell content in fractionated and unfractionated PCS

Table 3 shows that in fractionated as well as in unfractionated PCS there was a highly positive correlation between the total histamine content and mast-cell concentration in the samples. The correlation coefficients were 0.96 and 0 93 respectively The total material showed a correlation coefficient of 0.91

There was no positive correlation between the total histamine content and the leucocyte concentration either in fractionated or in unfractionated PCS

The histamine content per mast cell in the fractionated and unfractionated PCS was calculated to be 39 and 28 μg histamine base per 10^6 mast

Table 3

Total histamine content as well as mast-cell and leukocyte counts in unfractionated peritoneal cell suspensions (PCS) and fractionated peritoneal cell suspensions (FPCS) from rats sensitized to horse serum. Histamine release determined following incubation with horse serum.

Pool number		μg histamine base per ml	Mast cells per μl	Leucocytes per μl	μg histamine base per 10^6 mast cells	% histamine released by horse serum
	1	0.82	39	2900	21	59
	2	1.86	61	3360	31	23
PCS	3	0.64	16	2832	40	43
	4	2.70	131	1572	21	45
	5	0.51	20	4200	26	36
mean					28	41
	1	0.17	4	10	42	12
	2	0.24	16	16	15	9
FPCS	3	0.08	3	33	31	11
	4	1.83	40	40	46	21
	5	0.95	15	10	62	19
mean					39	14

Table 4

Differential counts of leucocytes in fractionated peritoneal cell suspensions (FPCS) and unfractionated peritoneal cell suspensions (PCS) from rats sensitized to horse serum. The figures indicate percentage of cells.

Pool number		Granulocytes				Lymphocytes	Monocytes
		Polymorphonuclear neutrophils		Eosinophilic	Basophilic		
		immature	mature				
	1	1	0	11	0	87	1
FPCS	2	8	1	26	0	62	3
	3	1	0	18	0	80	1
	1	10	0	21	0	67	2
PCS	2	9	0	18	0	72	1
	3	1	1	21	0	77	0

Table 5

Release of histamine by horse serum in suspensions of peritoneal cells from rats sensitized to horse serum. The peritoneal cells were pre-treated with 35 % (w/v) bovine serumalbumin or with modified Tyrode solution at 21 for 20 min. Thereafter they were transferred to modified Tyrode solution and incubated with horse serum.

PCS from rat no.	Per cent histamine released — Pre- treatment: serum albumin	modified Tyrode
1	37	64
2	33	70
3	10	33
4	9	36

cells. There was no significant difference between these values (the t test showing $P > 0\ 1$).

The histamine release in the suspensions was investigated after incubation with horse serum. In the fractionated PCS around 14 / of the total content and in the unfractionated PCS 41 / was released. These values are significantly different ($P < 0\ 01$).

Table 6

Lacking liberation of mast-cell disrupting factor in peritoneal cell suspensions from horse serum-sensitized rats following incubation with horse serum. This is expressed by the lacking ability of the supernatant to release histamine in peritoneal cell suspensions from non-sensitized and from egg albumin sensitized rats. Amount of released histamine expressed in per cent of the total content of the sample. Three determinations were done on each sample.

Per cent free histamine released by addition of: supernatant to PCS from rats — non-sensitized	supernatant to PCS from rats — sensitized to egg albumin	mod. Tyrode to PCS from rats — non-sensitized	mod. Tyrode to PCS from rats — sensitized to egg albumin
0	0	1	0
0	1	3	3
4	3	3	4

Role of fractionation medium

The release of histamine from those peritoneal cells which had been suspended in bovine serum albumin before incubation with horse serum in a modified Tyrode solution, was reduced by 50–75 / (table 5)

Possible liberation of a mast-cell disrupting factor

Following incubation of PCS from horse serum-sensitized rats with horse serum, the cell free supernatant released a maximum of 4 % of the total histamine content in the PCS both from non-sensitized rats and from rats sensitized to egg albumin (table 6). The same degree of histamine release was obtained when these PCS were incubated with a modified Tyrode solution, instead of the above mentioned supernatant (table 6).

Discussion

On incubation with specific antigen of peritoneal cell suspensions (non-isolated mast cells) from horse serum-sensitized rats, the release of histamine occurred within 30 sec. Therefore, the antigenic histamine release is a rapid process. This is in keeping with results obtained in the study of isolated peritoneal mast cells from rats (PERERA & MONGAR 1963).

The proportion of released histamine is independent of the number of mast cells in the cell suspension. The variations in the mast cell count from cell suspension to cell suspension are thus of no significance in the histamine release.

Histamine release increases with the logarithm of the antigen concentration, and this increase appears to be rectilinear (fig. 1) This finding is at variance with that of GARCIA AROCHA (1961) who observed a release only when the horse serum concentration was 5 %. However other investigators, studying isolated peritoneal mast cells, have found a histamine release at 1 / (UVNÄS & THON 1959) as well as at 20 / horse serum (PERERA & MONGAR 1963)

To ascertain whether the histamine content is derived exclusively from the mast cells, the correlation between histamine and mast-cell content was investigated in those suspensions in which the ratio mast cell leucocyte was about 3 100 and also in fractionated samples of these suspensions, with fewer leucocytes (ratio from 1 10 to 15 10). Fractionated as well as unfractionated peritoneal cell suspensions showed a highly positive correlation between the histamine and mast-cell content, but no positive correlation between histamine and leucocyte content. Furthermore, both categories showed the same percental distribution of leuco-

cytes (table 4). Thus, the histamine content must be derived exclusively or predominantly from the mast cells.

No significant difference was found in the total histamine content per mast cell between fractionated and unfractionated peritoneal cell suspensions (39 and 28 μg/10⁶ mast cells respectively). This indicates that the distribution of mast cells with a high and a low histamine content is the same in both categories of suspension.

It is striking that on incubation with specific antigen a smaller percentage of the total histamine content was released from the fractionated than from the unfractionated peritoneal cell suspensions (table 3). Thus, fractionation of the cells by means of the fractionation medium bovine serum albumin reduces the sensitivity of the mast cells to the antigen. Similar findings were made by JOHNSON & MORAN (1966), using other media. The explanation may be either that particularly resistant mast cells make their way into the fractionation medium or that this medium affects the mast cells in a way which reduces their sensitivity. The latter explanation was substantiated by an experiment showing that after being left for a short time in bovine serum albumin, the peritoneal cells release less histamine (table 5). This explanation has recently been advanced for the reduced sensitivity of mast cells to the histamine liberator compound 48/80 in certain fractionation procedures (NORN 1967a). One should, therefore, think twice before fractionating the mast cells by means of an fractionation medium, if the mast cells to be investigated are to be biologically intact.

The antigen-antibody reaction may also damage cells other than the mast cells. It may, for instance, cause lysis of polymorphonuclear leucocytes (WAKSMAN 1953) and disruption of eosinophilic leucocytes (GARCIA AROCHA 1961). SEEGERS & JANOFF (1966) have demonstrated that rabbit exudate polymorphonuclear leucocytes contain a mast-cell disrupting factor. It cannot be ruled out, therefore, that other cells or substances, apart from the mast cells in the peritoneal cell suspension, may influence the histamine release from the mast cell by liberating, in the course of the antigen-antibody reaction, a factor which then causes histamine release from the mast cell. The possible presence of a liberated mast-cell disrupting factor was therefore investigated in the peritoneal cell suspension by studying the ability of the cell-free supernatant to release histamine in a peritoneal cell suspension from non-sensitized rats as well as from rats sensitized to a non-specific antigen (egg albumin). As no histamine release was demonstrated, such a factor is probably not liberated. In this connection the author disregarded a possibility which does not appear particularly likely, viz. that this factor may in fact be liberated and that, after exerting its effect, it is immediately bound or inactivated

so completely that it is no longer demonstrable It is likely therefore, that the antigen has a histamine-liberating effect by reacting exclusively with the mast cell, other cells or substances in the peritoneal cell suspension being of no significance in the histamine release. In experimental studies of this nature it is, therefore, better to study non fractionated than fractionated mast cells, as the fractionation medium may alter the sensitivity of the cells to the antigen.

Summary

Histamine release was studied in suspensions of non-isolated peritoneal mast cells from sensitized rats following *in vitro* incubation with specific antigen.

The release of histamine takes place within 30 sec. It is independent of the mast-cell concentration, but apparently increases in proportion to the logarithm of the antigen concentration.

A highly positive correlation between histamine and mast-cell content in these suspensions as well as in fractionated samples of these suspensions containing fewer leucocytes, show that the histamine must be derived almost exclusively from the mast cells.

The presence of a liberated, mast-cell disrupting factor could not be demonstrated in the cell suspension after incubation. Hence, it is likely that the antigen releases histamine by reacting only with the mast cell, the other cells and substances having no influence on the histamine release.

Fractionation of the cells by means of the fractionation medium bovine serum albumin reduces the sensitivity of the mast cells to antigen The importance of studying non fractionated mast cells is emphasized

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The doses of amphetamine refer to D-amphetamine sulphate.

I addition to amphetamine, the following drugs were used. All doses refer to the respective preparations.

Anticholinergics l-hyoscyamine sulphate, atropine sulphate, scopolamine hydrochloride (Sigma), benzhexol hydrochloride (Artane ® Lederle), benactyzine (Suavitil ® Dumex), caramiphen (Parpanit-Wirksubstanz, Geigy), N-methylatropinium nitrate.

Cholinergics. Physostigmine salicylate, oxotremorine sesquifumarate (Aldrich), arecoline bromide.

Other drugs Perphenazine (Trilafon Injectable, Schering) and as α-methyltyrosine was used α-methyl-p-tyrosine methylester hydrochloride (H 44/68, Hässle).

I the experiments of fig. 1 nd table 1 amphetamine and an anticholinergic drug or placebo (all solutions made isotonic with NaCl) were given simultaneously and each rat was bserved for five minutes period starting 50 min. after the injections. In preliminary experiments the peak effect of the drugs used was seen at approximately this time after the injection. The rats were injected at intervals f 7 min., so that the observer could always concentrate his attention on one rat only 14 rats were observed daily so that each of the experiments lasted about week. In the test experiments 1 3 the rats were assigned to the various treatments according to schedule of randomization, which was unknown to the observer Each rat was used once only Continuous sniffing as recorded in table 2 refers to sniffing in the air as well as at the cage wire netting it excludes such activities as eating and grooming (few rapid strokes over the snout were allowed) but locomotion.

Continuous sniffing at the cage wire netting was recorded seperately Grooming activity was also recorded and in experiment 1 nd 2, separation was made between the three main forms grooming with the forelegs, the mouth and the hind legs (RANDRUP & MUNKVAD 1965). The weights of the rats used for these experiments varied between 165 and 265 g only

Results

Behavioural effects of amphetamine in various doses

Sniffing and grooming of rats treated with doses of amphetamine varying from 0.9 to 5 1 mg/kg were observed as described above.

The graph in fig. 1 shows the number of rats showing continuous sniffing during the 5 minutes observation period It will be seen that the ED_{50} for this response is around 1 7 mg/kg (Serum Institute strain) With lower doses sniffing there was in the air as at the cage wire netting but sniffing at the wires gradually became dominant, and was the only form seen with the two highest doses. At the same time the area of the cage sniffed at became restricted.

The observations on grooming confirmed that this form of activity was diminished by amphetamine (RANDRUP & MUNKVAD 1965). With the

) In previous papers w have referred to d-amphetamine base, using an apparently erroneous conversion factor between salt and base. What was formerly reported as 3 mg/kg base correctly corresponds to 5 mg sulphate/kg and is the standard dose for the production of stereotypy in the Serum Institute strain. For the Wistar strain 10 mg/kg is used.

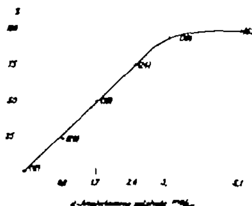


Fig. 1 Per cent of rats sniffing continuously during the observation period 50–55 minutes after injection of various doses of amphetamine.
The numbers of rats injected with each dose are shown in parenthesis in the graph Further details in table 1 and "Methods" section.

lowest dose grooming was observed in all 29 rats but one, and in 5 out of 17 rats all the three main forms of grooming (with the forelegs, with the mouth and with the hind legs) were observed Two forms of grooming were still observed in 3 out of 12 rats with 1 7 mg/kg. With 2.6 mg/kg proper grooming in one form was seen in 3/11 rats, but with the higher doses only a few abortive attempts at grooming occurred.

With increasing dose of amphetamine locomotion, eating and drinking disappeared concurrently with the grooming, so that the behavioural repertoire of the rats gradually became more and more restricted. Finally with the highest doses the behaviour acquired a very stereotyped character consisting mainly of continuous sniffing accompanied by licking or biting. The sniffing was restricted to a small area of the cage floor or lowest part of the walls. The rats sat in a crouched posture pressing their body against the wall. Backward locomotion occurred occasionally but not normal forward locomotion. This extremely stereotyped behaviour has been described in detail in previous publications (RANDRUP MUNKVAD & UDSEN 1963 RANDRUP & MUNKVAD 1965 MUNKVAD & RANDRUP 1966).

Behavioural effects of anticholinergics

Like amphetamine, the anticholinergics had an excitatory effect on rats and induced much sniffing. The sniffing did not, however become completely continuous even after high doses, and licking or biting were exceptional. The head was kept quiet for short periods, and grooming (in all three forms) occurred occasionally Eating was also seen. Rearing

with sniffing at the upper part of the cage and some forward locomotion occurred in all animals. Crouching in a corner sniffing at the cage floor and backward locomotion were uncommon. Spells of rapid forward running were sometimes elicited by a sudden noise. The behaviour elicited by the anticholinergics is thus reminiscent of that seen after the smaller doses of amphetamine. In both cases there is increased motor activity with sniffing, locomotion and rearing as the most predominant features. The more extreme forms of stereotypy which are seen after higher doses of amphetamine cannot, however be produced by anticholinergics. Thus we found by direct comparison, that there was little difference between rats given 10 or 90 mg/kg of benzhexol.

The "anticholinergic" form of excitation was observed after 1-hyoscyamine (12 and 37 mg/kg, 4 rats 1 and 3 mg/kg had little effect, 4 rats), atropine (dl-hyoscyamine 95 mg/kg, 2 rats animals with 5–35 mg/kg (6 rats) or 190–380 mg/kg (4 rats) were more quiet, scopolamine (1 3 10 27 and 50 mg/kg, 27 rats 0.15 mg/kg had less effect, 2 rats), benzhexl (5 10, 20 30 and 90 mg/kg, 26 rats), benactyzine (6 and 30 mg/kg, 4 rats 1 mg/kg had little effect, 2 rats) and caramiphen (5 15 and 50 mg/kg, 6 rats) These observations were made in preliminary experiments and in the experiments included in table 2, in which the animals were observed for 2 hours or until the effect of the drug had clearly declined. The effect was maximal around one hour after the injection. Confirmatory evidence was found in the experiments reported in table 1 where the rats were observed for 5 minutes only starting 50 minutes after the drug injection.

Combinations of anticholinergics and amphetamine

Table 1 shows that continuous sniffing can be produced by 0.9 mg/kg amphetamine in combination with any of the anticholinergics except methylatropine, which is a quaternary amine and therefore only passes the blood brain barrier to a very small extent. With the highest doses of the anticholinergics combined with 0.9 mg/kg amphetamine the sniffing was mostly or exclusively at the wire netting. When the drugs were given separately continuous sniffing in more than 50 per cent of the animals or continuous sniffing at cage wires is produced only by higher doses of amphetamine and not by any dose of the anticholinergics (see also table 2).

Other behavioural effects of higher doses of amphetamine, such as complete inhibition of forward locomotion pressing the body against the cage wall, licking and biting of cage wires were not seen in the experiments with anticholinergics in combination with the low dose of amphetamine reported in table 1 All these features were produced, however when anticholinergics were given to rats, in which the behavioural effects of a

Table 1

Potentiation of amphetamine-induced sniffing by anticholinergic drugs. Three separate experiments and preliminary experiment were made. For details of procedure, see Methods section.

d Amphetamine sulphate mg/kg	Anticholinergic drug mg/kg	Rats sniffing continuously/Rats injected				
		Prelim. Exper	Exper 1	Exper 2	Exper 3	Exper 1–3 Combined
0.9		0/8	2/11	1/6	4/12	7/29
1.7				5/12	4/6	9/18
2.6			9/12		9/12	18/24
3.4		8/0		12/12	5/6	17/18
5.1					6/6	6/6
0					0/12	0/12
	l-Hyoscyamine					
0.9	3.1				3/6	3/6
0.9	12.4	3/4	5/6	6/6	4/6	15/18
0.9	24.8	4/4				
0	12.4	0/4				
0	24.8				1/6	1/6
	Scopolamine					
0.9	0.14			6/6	3/6	9/12
0.9	0.56	4/4	6/6		4/6	10/12
0.9	2.2			6/6		6/6
0	0.56	0/4				
0	1.1				1/6	1/6
	Benzhexol					
0.9	1.15			5/6	0/6	5/12
0.9	5	4/4	6/6		2/6	8/12
0.9	10	4/4				
0.9	20			6/6		6/6
0	5	0/4				
0	10				0/6	0/6
	Benactyzine					
0.9	2.5				1/6	1/6
0.9	5	4/4				
0.9	10	4/4	6/6	6/6	5/6	17/18
0.9	15	4/4				
0	10	0/4				
0	20				2/6	2/6
	Caramiphen					
0.9	2.5				2/6	2/6
0.9	5	2/4				
0.9	10	4/4	5/6		4/6	9/12
0.9	15	4/4				
0.9	20	3/4				
0	10	0/4				
	Methylatropine					
0.9	24		0/6		0/6	0/12

Results differing significantly ($P < 0.01$) from the result with 0.9 mg/kg amphetamine alone in the same vertical column. Statistical evaluation by Fisher's exact probability test (SIEGEL 1956, see p 96f).

Table 2.

Characteristic features of amphetamine-stereotypy inhibited by perphenazine and restored by anticholinergic drugs.

Perphenazine was given 45 min. before amphetamine and anticholinergic (or placebo) The rats were bserved for 2 hrs. or more after the latter injection. 6–12 rats were observed simultaneously on each experimental day always including 2 rats treated with amphetamine plus perphenazine only

Treatment				Behaviour: rats performing the resp. behaviour / rats injected		
Anticholinergics: Drug	Dose mg/kg	Perphenazine 0.1 mg/kg	Amphetamine 10 mg/kg	Continuous sniffing t cage wires	Biting or licking	Characteristic amphetamine posture[1])
–	–	+	+	0/12	0/12	0/12
Scopolamine	3	+	+	6/6	5/6	6/6
Benzhexol	10	+	+	5/6	5/6	3/6
–	30	+	+	6/6	6/6	4/6[2])
Scopolamine	3	+		0/6	0/6	0/6
Benzhexol	10	+	–	0/6	0/6	0/6
–	30	+	–	0/6	0/6	0/6
Scopolamine	3	–		0/6	0/6	0/6
Benzhexol	10	–	–	0/6	1/6[3])	0/6
–	30		–	0/6	0/6	0 6
Scopolamine	27	–	–	0/6	2/6[3])	0/6
Benzhexol	90	–		0/6	4/6[3])	1/6

[1]) Pressing of body against cage wall or corner head downwards, no forward locomotion (In rats treated with amphetamine + anticholinergics hort spells of rapid forward running may be elicited by sudden noise)

[2]) One of these animals performed backward locomotion, the only one in this table.

[3]) Only occasional.

higher dose of amphetamine had been inhibited by perphenazine. These results are given in table 2.

α Methyltyrosine an inhibitor of catecholamine syntheses (CORRODI & HANSON 1966), inhibited amphetamine-stereotypies in a manner similar to that of perphenazine (RANDRUP & MUNKVAD 1966). In a few experiments (4 rats) we inhibited amphetamine by this drug (doses 200 and 300 mg/kg)

Table 3

Antagonistic effect of cholinergic drugs against amphetamine-induced stereotyped behaviour.

All rats were pretreated with 20 mg/kg α-methyltyrosine followed after two hours by 10 mg/kg D-amphetamine sulphate. Physostigmine and oxotremorine were injected at the same time as amphetamine, arecoline 40 and gain 50 min. later

Cholinergic drug	Doses mg/kg	No of rats	Behaviour
None		6	Stereotyped continuous sniffing at cage wires, little locomotion no grooming[1])
Physostigmin	0.4	6	very little sniffing at cage wires head quiet for long periods, locomotion now and then. Several times grooming with the forelegs and the mouth.
Oxotremorine.	2	6	Similar to the behaviour after physostigmine but more locomotion and less grooming.
Arecoline	10 + 10	6	Similar to the behaviour after oxotremorine. Peak effect of arecoline 10–20 min. after the last injection, then gradual return of stereotypies.

[1]) This behaviour was displayed by all the 6 rats from 50 min. to 1 h 50 min. after amphetamine; the observations of the behaviour after amphetamine + the cholinergic drugs were all done in this time interval. The α-methyltyrosine shortened the period of stereotypy but the dose was too small for complete.

and as in the perphenazine inhibition experiments of table 2 we were able to restore the amphetaminebehaviour by benzhexol (30 mg/kg).

Combinations of cholinergics and amphetamine

The results with anticholinergics suggest that these substances would have an antagonistic action against amphetamine-stereotypies. In preliminary experiments we also observed some antagonistic action, but this was only partial (small head movements continued although sniffing at cage wires was abolished) and temporary In addition, the behavioural observations were obscured by tremor and an apparent general state of illness of the rats. Similar observations were made by HALLIWELL (1964). SCHELKUNOV (1964 & 1967) found that arecoline produced a short lasting dis-

appearance of amphetamine stereotypies of rats. An idea for prolonging the effect of the cholinergic drugs was, however suggested to us by the work of GOLDBERG & JOHNSON (1964). These investigators studied the inhibitory effect of chlorpromazine and cholinergics on discrete avoidance behaviour of rats, and found it considerably greater than additive prolongation of this effect, when the drugs were combined. In preliminary experiments we also found a marked potentiation and prolongation of the anti-stereotypy effect, when the cholinergics were combined with small subeffective doses of other amphetamine antagonists, such as perphenazine or α-methyltyrosine. In table 3 some experiments are recorded, in which the opportunities for behavioural observations were particularly favourable since the anti-amphetamine effect was still marked, when other cholinergic effects (tremor lacrimation, etc.) had subsided.

Discussion

The literature cited in the introduction indicates that the stereotyped hyperactivity is associated with dopamine in the corpus striatum of the brain, the effect of this amine being increased by amphetamine and reduced (by inhibition of synthesis) by α-methyltyrosine Other evidence indicates that perphenazine and related neuroleptic drugs also diminish dopamine activity perhaps by blockade of dopamine-sensitive receptors in the brain. (HORNYKIEWICZ 1966 NYBÄCK *et al* 1967)

The evidence presented in this paper shows that the effects of these drugs are dependent on the state of a cholinergic system and this indicates the existence in the brain of a dopaminergic-cholinergic balance influencing such behavioural features as motor activity level and stereotypy (or perseveration).

Many behavioural effects of amphetamine are reported in the literature. Some of these may be due to the action of the drug on brain dopamine, while others (e.g. increased locomotion) seem to be more related to an action on noradrenaline in the brain (RANDRUP & SCHEEL KRÜGER 1966 SCHEEL KRÜGER & RANDRUP 1967).

Besides the stereotypy several other effects of amphetamine have been found to be enhanced by anticholinergics. These include increased mobility of mice (TRIPOD 1952 GALAMBOS *et al* 1967) disturbance of conditioned reflexes of rats in a labyrinth (SCHELKUNOV 1963 & 1967), increase of operant shock-avoidance behaviour of rats (CARLTON 1963) and toxicity in aggregated mice (MORPURGO & THEOBALD 1964 MENNEAR 1964) CARLTON (1963) found that amphetamine as well as atropine and scopolamine produced various features of perseveration in operant behaviour

of rats. Among these investigators GALAMBOS *et al* SCHELKUNOV and MENNEAR found that the amphetamine effects antagonized by cholinergic drugs (physostigmine, tremorine) BARNES (1964 & 1966) found anti-sleep and EEG effects of amphetamine antagonized by physostigmine In a recent review of research on the extrapyramidal motor system HORNYKIEWICZ (1968) concludes that dopamine and acetylcholine act as mutual antagonists with regard to their influence on the functioning of the extrapyramidal centres. Other aspects of adrenergic-cholinergic balance systems in the brain have been discussed by SCHELKUNOV (1967)

In some experiments, however amphetamine and anticholinergics were found to produce different and antagonistic effects. Amphetamine inhibited the "amnesic" effect of scopolamine in rats (PAZZAGLI & PEPEU 1964) and disturbances of various conditioned reflexes in rats, rabbits and cats produced by anticholinergics (MICHELSON 1961 LINUCHEV *et al* 1961) The two latter investigators also found that caramiphen-induced mental disturbances of humans (impairment in multiplication-tests, memory-tests, etc.) were reduced by amphetamine. WHITE *et al* (1961) report that several behavioural effects of atropine and scopolamine in *dogs and monkeys are counteracted by amphetamine and also confirm* the earlier finding that the EEG desynchronization effects of amphetamine are prevented by anticholingergics.

The interaction between amphetamine and cholinergic systems in the brain is therefore not a simple one but it seems reasonable to assume that the above-mentioned dopaminergic-cholinergic balance is involved in some of the cases in which amphetamine effects are enhanced by anticholinergics and antagonized by cholinergics.

Discussion

In various experimental situations amphetamine induced stereotyped hyperactivity of rats was found to be enhanced by anticholinergic drugs and antagonized by cholinergics. Previously the stereotyped behaviour was related to an effect of amphetamine on brain dopamine, and the findings therefore indicate the existence in the brain of a dopaminergic-cholinergic balance which influences such behavioural features as motor activity level and stereotypy (or perseveration)

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Determination of Prekallikrein in Human Plasma

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In a previous paper the release of kinin by acetone in a human plasma substrate was examined, and experimental conditions yielding a plasma kallikrein preparation with high activity were established (BRISEID ARNTZEN & DYRUD 1968). In the present work a method for determination of the kallikrein activity is described based on the release of kinin in citrated human plasma. The technique was used in plasma specimens from 13 males.

Technique

A. *Materials and Assays*

Plasma substrate Human blood was collected by venepuncture into siliconized, gradu ted cylinder containing 1 ml of a 3.1 % sodium citrate dihydrate solution per 9 ml of blood, transferred to siliconized centrifuge tube, and centrifuged t 1 3 × 10^3 g for 30 minutes t 10 Then was added 4 0 mg of EDTA-2N dissolved in 0 04 ml f water per ml of citrated plasma, with subsequent heating for 30 minutes at 37 The substrate was stored at −20 as 1 ml samples corresponding to 0.81 ml plasma The pooled plasma from at least 3 men was used for the preparation.

Plasma kallikrein. Citrated plasma was prepared as described bov for the plasma substrate (EDTA-treatment omitted) and stored at −20 For kallikrein activation, 0.20 ml acetone was added per ml citrated plasma (16.7 % (/v) of acetone), and the mixture left for about 17 hours at room temperature (22 ± 2°). The kininase activity was then eliminated by incubation with EDTA as stated above, and the kininogenase preparation was ready for assay

A kallikrein standard preparation used f the estimation of kallikrein activities in different plasma specimens was based on the pooled plasma from 3 men.

Assays. The kinin determinations are carried out on the isolated rat uterus as "bracket ing assays with dose ratio of 3 2. Bradykinin was used as standard substance.

B. *Methods*

Determination of plasma kallikrein. T each of 4 one-ml samples of plasma substrate 0.14 ml of acetone and then 0 015, 0.030, 0.045 and 0.060 ml of kallikrein preparation respec

tively were added. To one sample 0.080 ml acetone and 1.00 ml of kallikrein preparation were added. A blind test with 0.14 ml of acetone, but without any enzyme added, was used as a control. After incubation at 37° for exactly 10 minutes 0.20 ml samples were withdrawn and the reaction stopped by heating for 5 minutes in a boiling waterbath after dilution with 2.5 ml of saline. The samples were kept at 4° and assayed on the same day.

The kinin values were calculated as µg bradykinin/ml plasma, and the amounts obtained with the 4 submaximum kallikrein concentrations were converted to percentages of the amount obtained with excess of enzyme preparation, i.e. 1 ml/ml substrate. A concentration-effect curve was drawn and the amount of kallikrein corresponding to 50% release was estimated. The kallikrein activities in different enzyme preparations were calculated in terms of a standard kallikrein preparation assayed in parallel.

Comments on the Technique

A. *Materials*

Plasma substrate. The same procedure for the preparation of plasma substrate was previously used by BRISEID, DYRUD & RINVIK (1967) and BJELTVEDT & BRISEID (1967). When an excess amount of plasma kallikrein preparation was added to plasma substrate specimens, about ⅓ of the kinin was released, while complete exhaustion of the kininogen required the presence of acetone (table 2). This is in accordance with the observations of MARGOLIS & BISHOP (1963). JACOBSEN (1966a & b) also obtained release values of about 30 % by the addition of plasma kallikrein to human plasma. The last-mentioned author described the preparation of 2 kininogen fractions, substrate 1 and substrate 2 (about ⅓ and ⅔ of the total kininogen respectively) and showed that his plasma kallikrein released kinin from substrate 1 only, while glandular kallikreins also reacted with substrate 2.

Plasma kallikrein. According to previous experiments (BRISEID, ARNTZEN & DYRUD 1968) the acetone concentration and the activation time used should bring about a maximum yield of kininogenase activity. In the present work control experiments showed that only traces of kininogen could be detected after the 17 hour activation period. This is in accordance with data published by MARGOLIS & BISHOP (1963).

B. *Methods*

Determination of plasma kallikrein. The acetone, which was added to a final concentration of about 12.5 % (v/v), inhibited the kallikrein inhibitors, but at the same time also inhibited plasma kallikrein itself. Fig. 1 shows one concentration-effect curve based on experiments with acetone (A) and another based on experiments without addition of acetone (B). The enzyme concentration required for a 50 % kinin release was about 30 % higher when acetone was present, 68 µl against 52 µl enzyme pre-

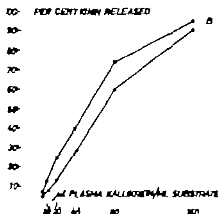


Fig 1 *Plasma kallikrein concentration effect curves.*
Plasma substrate: Human citrated plasma, stabilized with EDTA 2Na, 4 mg/ml plasma.
Kallikrein preparation Human citrated plasma activated with acetone 16.7% (v/v), for 17 hours at room temperature before addition of EDTA 2Na.
Curve A Acetone added to concentration of 12.5 % (v/v) before incubation with kallikrein.
Curve B: No acetone added.
Incubation time 10 minutes.
Kinin released calculated as μg bradykinin/ml plasma
Kinin released with excess of kallikrein 1 6 μg/ml plasma.
For further details see text.

paration being required per ml of plasma substrate This means that the enzyme was more strongly inhibited than the inhibitors, when a 10-minute incubation period was used. It has been shown (BRISEID ARNTZEN & DYRUD 1968) that a 1 hour s contact period with 12 % (v/v) acetone was not sufficient to obtain a complete inactivation of the inhibitors. A reversible inhibition however takes place rapidly and accordingly no preincubation period with acetone was suggested.

Fig. 2 demonstrates time effect relationships An enzyme concentration was used which caused about 50 % kinin release when no acetone was present. Curve B shows that a maximum release effect was obtained after incubation for about 6 minutes. When 12.5 % (v/v) acetone was added (curve A), the rate of release was considerably slower but the release process continued beyond 6 minutes, indicating the elimination by acetone of kallikrein inhibitors. After incubation for 14 to 15 minutes the release was 50 % of the maximum (obtainable without acetone) in both experiments. Control tests without any kallikrein showed that the acetone-induced kinin release was negligible when incubation periods of 16 minutes or less were used

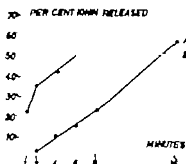


Fig. . *Plasma kallikrein time effect curves.*
Plasma substrate and kallikrein preparation, see fig 1
Curve A Acetone dded to a concentration of 12.5% (/).
Curve B No acetone added.
Amount f kallikrein preparation/ml plasma substrate 50 µl.
Kinin released calculated as µg bradykinin/ml plasma.
F further details see text.

Reproducibility The "standard-column" in table 1 demonstrates the precision of determinations of the amount of kallikrein preparation corresponding to 50 / kinin release of that obtained with excess of enzyme. Thirteen different samples of the same batch of plasma kallikrein were assayed against 13 different samples of the same batch of plasma substrate, at different times. The releases of kinin observed in these assays with excess amount of kallikrein preparation (see Methods) ranged from 1.2 to 1 5 µg/ml plasma, calculated as bradykinin and with an average of 1 4 µg/ml plasma. Controls incubated in parallel without addition of kallikrein showed kinin releases too low to be determined in 5 assays, and ranging from 0 05 to 0 10 µg/ml plasma, calculated as bradykinin in the remainder of the assays, and were not taken into account.

Results

Table 1 shows the results of determinations of acetone-activated plasma kallikrein in plasma specimens from 13 healthy males in the age range 23 to 37 years. It should be mentioned that a questionnaire was sent out to all the subjects examined in order to check up on any possible allergic disposition or diseases (BRISEID DYRUD & LANGE-NIELSEN, in press) Subject number 4 who was 30 years old had a chronic bronchitis of probable allergic origin in the period 1939 to 1950.

Table 2 gives the results of determinations of kininogen in the same plasma specimens as were examined for prekallikrein. The table shows the amounts of kinin, calculated as bradykinin/ml plasma, released by acetone-activated plasma kallikrein, probably corresponding to JACOBSEN's sub-

Table 1

Determination of prekallikrein in plasma specimens from 13 men.

The individual kallikrein preparations were tested for kinin releasing activity in comparison with standard kallikrein preparation. The same batch of plasma kininogen substrate was used in all tests. For experimental details and *f information about subject 4 see text.

Subject number	µl kallikrein preparation per ml plasma substrate causing 50 % kinin release		
	Test	Standard	Relative activity Test/Standard
1	30	30	1.0
2	30	45	1.5
3	80	50	0.6
4	15	40	2.7
5	45	50	1.1
6	20	30	1.5
7	50	35	0.7
8	45	45	1.0
9	55	50	0.9
10	40	50	1.3
11	75	35	0.5
12	40	30	0.8
13	30	30	1.0
Mean	42.7	40.0	1.12
S.D	19.2 (45.0 %)	8.7 (21.8 %)	0.57 (50.9 %)

strate 1 (1966a & b JACOBSEN & KRIZ 1967), as well as the maximum amounts of kinin releasable with acetone (BRISEID DYRUD & RINVIK 1967), corresponding to the total kininogen. The kinin values were 1.5 µg and 4.8 µg/ml plasma respectively and the minor kininogen fraction accordingly about ⅓ of the total kininogen.

Discussion

In the present paper the term plasma kallikrein has been used to indicate all plasma kininogenases activated by acetone. The inactive enzyme precursors were called prekallikrein.

The plasma kallikrein preparation used contained kininogenases capable of causing complete exhaustion of the kininogen provided that acetone was added to suppress kallikrein inhibitors, and provided incubation period was not too short. The kallikrein

Table 2

Determination of total kininogen and the kininogen exhausted by plasma kallikrein in plasma specimens from 13 men.

I Total kininogen determined by the acetone activation method (BRISEID, DYRUD & RINVIK 1967).

II The minor kininogen fraction determined by the kinin release caused by excess of plasma kallikrein preparation.

For details see text.

Subject number	Kinin released/ml plasma as µg bradykinin		II/I
	I	II	%
1–4	5.4	1.4	[illegible]6
5–7	4.7	1.6	34
8	5.0	1.7	34
9	4.6	1.4	30
10	4.3	1.1	26
11	4.9	1.7	35
12	4.7	1.7	36
13	4.7	1.7	36
Mean	4.79	1.54	3[illegible]1

were carried out in the presence of 12.5 / (v/v) acetone to eliminate the effect of kallikrein inhibitors present in the substrate. However the short incubation period used only allowed the release of kinin from a minor kininogen fraction. It was shown that the release of kinin from this fraction, probably corresponding to substrate I of JACOBSEN (1966a & b JACOBSEN & KRIZ 1967) took place in a few minutes, while the exhaustion of the main kininogen according to previous experiments (BRISEID ARNTZEN & DYRUD 1968) required an incubation period of about 4 hours.

The fact that the kallikrein preparation selectively released kinin from the minor kininogen fraction when acetone was not present, probably reflects the above-mentioned difference in reaction rate between the releasing enzyme and each of the two kininogen fractions. Thus, if the inhibitors are not suppressed by acetone, the kallikrein will be inactive at a time when only insignificant amounts of kinin have been released from the major kininogen If the kallikrein inhibitors were inactivated by heating plasma substrate at 60° for 1 hour then full kinin release was obtained with excess of plasma kallikrein without the addition of acetone (BRISEID ARNTZEN & DYRUD unpublished experiments). The rate of release, however was reduced for both kininogen fractions, probably suggesting some denaturation of the substrate.

It can be concluded that the method described will assay all plasma

kininogenases activated by acetone and capable of releasing kinin from a minor fast reacting kininogen fraction. Interference from acetone susceptible inhibitors was eliminated The incubation period used was too short to allow a significant acetone induced activation of the pre kallikrein of the plasma substrate used

Summary

A method has been described for the determination of prekallikrein in human plasma. The procedure included activation of prekallikrein with 16 7 % (v/v) acetone, release of kinin in a human plasma substrate by the active kallikrein, and subsequent assay of the released kinin on the isolated rat uterus. The interference from kallikrein inhibitors in the plasma substrate was eliminated by incubation in the presence of 12.5 % (v/v) acetone. The amount of kallikrein preparation causing 50 % kinin release of that obtained with excess of enzyme was estimated and the activity then calculated on the basis of a standard kallikrein preparation assayed in parallel

The method was used to determine prekallikrein in plasma specimens from 13 healthy males. The average value of the amount of plasma kallikrein preparation causing a 50 % kinin release was 43 µl per ml plasma substrate with S.D = 19.2 (45 %). The determinations of 13 different samples of the standard kallikrein preparation gave an average value of 40 µl enzyme preparation per ml plasma substrate and the S D was calculated to be 8 7 (22 %)

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Studies on the Release of Kinin by Acetone in Human Plasma

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The activation of plasma kallikrein by acetone, first described by KRAUT FREY & WERLE (1933) has been used by several research workers. Most often the procedure has been adopted to provide an enzyme preparation, and relatively few papers give quantitative data about the activation process or the subsequent release of kinin (KRAUT FREY & WERLE 1933 FREY KRAUT & WERLE 1950 MARGOLIS & BISHOP 1962 & 1963 MARGOLIS 1966 BRISEID DYRUD & RINVIK 1967). The present work was carried out to obtain more detailed information about the activation of kinin-releasing enzymes and inactivation of inhibitors of such enzymes caused by acetone in human plasma. The experiments were based on determinations of the rate and extent of kinin release. The term "plasma kallikrein" has been consistently used in this paper to indicate all plasma kininogenases activated by acetone. The inactive enzyme precursors were called "prekallikrein" in deference to the suggestion of the committee on nomenclature for hypotensive peptides (WEBSTER 1966).

Technique

1 *Plasma substrat* was prepared as described by BRISEID DYRUD & ARNTZEN (1968). The pooled plasma from at least 3 men was used.

2. *Plasma kallikrein preparations.* Citrated plasma was prepared as described by BRISEID, DYRUD & ARNTZEN (1968) and in the different experiments was activated by incubation with varying amounts of acetone for varying periods f time t room temperature (22 ± 2°). The kininase activity was then eliminated by incubation for 30 mi utes at 37 with EDTA 2Na, 4 mg l 0.03 ml of water/ml citrated plasma. The pooled plasma from at least 3 men was used for all the *kallikrein preparations examined.*

3. *Determination of plasma kallikrein.* The kininogenase activity was tested by determining the amount of enzyme preparation required for 50% kinin release in the plasma substrate preparation, as described by BRISEID, DYRUD & AARSTEIN (1968).

4. *Assays.* The kinin determinations were carried out on the isolated rat uterus as "bracketing assays" with a dose ratio of 3 : 2. Bradykinin was used as standard substance. To eliminate day-to-day variations in the relative sensitivity of the rat uterus to bradykinin and the released kinins, the single results were calculated as percentages of a maximum kinin release value obtained by the acetone method of BRISEID, DYRUD & RINVIK (1967).

5. *Acetone evaporation.* In some experiments the acetone was removed from the incubates. This was carried out in siliconized, round-bottomed flasks at room temperature (22 ± 2°) under reduced pressure (5–10 mm Hg) on a rotating evaporator. An evaporation period of 30 minutes was used throughout. Control experiments showed that the evaporation procedure did not itself cause any kinin release.

Results

Significance of the acetone concentration for the release of kinin

Table 1 shows the results of 24-hour experiments carried out with different acetone concentrations and a substrate dilution of ½ (v/v). To obtain maximum kinin release in the observation period, at least 8% (v/v) acetone was required. The rate of release increased from 8% to 16% (v/v) acetone; then at 20% (v/v) acetone this was followed by a significant fall in kinin release. At this last concentration, the amount of kinin determined after incubation for 24 hours was as low as that obtained

Table 1

Significance of acetone concentration for the release of kinin.

Plasma substrate (EDTA 2Na, 4 mg/ml citrated plasma and 30 minutes at 37°) was diluted with mixtures of acetone and saline to a final dilution [illegible]f ½ (v/v) and incubated at 37°. Aliquots were diluted with saline, heated for 5 minutes at 100[illegible] and assayed on the isolated rat uterus. Kinin released was calculated as µg bradykinin/ml plasma. The percentages at 0, 2 and 6% acetone were based on an average maximum release value obtained in other experiments with the same substrate specimen.
For further details see text.

Acetone conc. ([illegible]/v)	0		2		6		8		10		14		16		20	
Kinin released	µg	%	µg	%	µg		µg	%	µg	%	µg	%	µg	%	µg	%
3 hours incub.	0.1	2	0.2	4	0.3	6	1.4	30	1.5	32	2.1	45	1.8	37	0.4	8
6 hours incub.	0.2	4	0.3	6	0.5	10	3.6	77	4.3	91	4.3	91	4.9	100	0.5	10
10 hours incub.	0.4	8	0.5	10	0.9	19	3.8	81	4.7	100	4.7	100	4.9	100	0.6	12
24 hours incub.	0.8	17	0.8	17	2.6	54	4.7	100	4.3	91	4.7	100	4.9	100	0.7	14

with 2% (v/v) acetone or that in the control experiment with no acetone added. Experiments with 16 / (v/v) acetone not shown in the table demonstrated that an *incubation period of about 4 hours was required* for maximum release of kinin.

In the experiments with 20 / (v/v) acetone some formation of pre cipitate took place during the first few hours of incubation In an attempt to discover whether loss of kallikrein in the precipitate might be responsible for the low kinin release values observed for 20 / acetone (table 1), an experiment was performed in which the sediment was centrifuged off *after 2½ hours incubation. This period should be sufficiently long to* allow inactivation of inhibitors of kallikrein (table 4) This was followed by acetone evaporation and further incubation. At the same time a parallel experiment was carried out without centrifugation. Table 2 shows that the rate of kinin release was significantly reduced when the precipitate was removed (columns I and IIb), but not to such an extent as to account for the low kinin release observed when acetone was contin *uously present (table 1) This must accordingly be due primarily to a* reversible acetone produced inhibition of factors present in the releasing system. A comparison of the kinin release values given in table 2 for 1 2½ and 12 hours acetone contact showed that part of the inhibition was

Table 2

Inhibition f kinin-releasing factors at 20% acetone

Plasma substrat diluted with a mixture of acetone nd saline to substrat dilution of ½ (/v) and an acetone concentration f 20% (v/v) was incubated at 37° acetone as evaporated and saline added t original volume. Then the mixture was further incubated and aliquots for assay withdrawn at various times.

I. The precipitat centrif ged off before cetone evaporation. Acetone contact period ½ hours.

II. No centrifugation. Acetone contact periods a, 1 hour b, 2½ hours c, 12 hours.

For further details see text nd table I

Kinin released	I		II		IIb		IIc	
	μg	%	μg	%	μg		μg	%
0 hours before evap	0.3	7	0.3	6	0.3	7	0.1[a]	2
0 hours after evap.	0.4	9	1 0	19	1 6	35	0.7	14
3 hours fter evap	1 7	37		–	3 7	80		
4½ hours after evap	–		4 6	88			1 4	28
7 hours after evap.	3 7	80	–		4.3	93	–	–
8½ hours after evap.	–		5 2	100		–	2.1	42
4 hours fter evap.	4.7	102	5.2	100	4 7	102	4.3	86

irreversible. It should be pointed out, however, that one cannot exclude the possibility that part of the slow release of kinin may also be due to the kininogen itself being altered by 20% (v/v) acetone.

Significance of the plasma dilution for the release of kinin caused by acetone

Table 3 shows the results of experiments carried out with different plasma substrate dilutions and a common acetone concentration of 10% (v/v). The rate of release increased with the concentration of plasma, as might be expected. For the substrate dilution of ¼ the release was about 50% after 6 hours and completed after 10 hours, which agrees well with the data previously given for the kininogen determination method based on acetone activation (BRISEID, DYRUD & RINVIK 1967).

Significance of the acetone concentration for the activation of kallikrein and inactivation of kallikrein inhibitors

Table 4 shows the results of 24-hour experiments with initial acetone concentrations ranging from 8 to 20% (v/v) and with a substrate dilution of ½ (v/v). After 1 hour's incubation the acetone was evaporated and incubation continued. It can be seen that 16% acetone is required to activate plasma kallikrein and to inactivate irreversibly the inhibitors

Table 3

Significance of plasma dilution for the release of kinin caused by acetone

Plasma substrate diluted with:
I. Acetone.
II. A mixture of acetone and saline to final substrate dilution of ½ (v/v).
III. A mixture of acetone and saline to final substrate dilution of ¼ (v/v).
Acetone concentration 10% (v/v) in all incubates.
For details see table 1 and text.

Incub. time hours	Kinin released/ml plasma as bradykinin					
	I		II		III	
	µg	%	µg	%	µg	%
2	2.9	56	1.5	29	0.7	13
3	4.5	87	2.6	90	1.7	33
6	5.2	100	4.6	88	3.0	58
10	5.2	100	5.2	100	5.2	100

Table 4

Significance of the acetone concentration for activation of kallikrein and inactivation of kallikrein inhibitors

Plasma substrate was diluted with mixtures of acetone and saline to a final dilution of ½ (v/v) (time 0). The mixtures were incubated at 37° for 1 hour, acetone was evaporated (30 minutes), and saline added to a substrate dilution of ½ (v/v). Then the mixtures were further incubated and aliquots for assay were withdrawn at various times.
For further details see table 1 and text.

Acetone conc. % (v/v)	8		12		16		20	
Kinin released after time in hours	μg	%	μg	%	μg	%	μg	%
1	0.4	8	0.2	5	0.5	12	0.3	6
1½	1.6	31	1.4	35	1.6	37	1.0	19
3	1.9	37	2.4	60	4.3	100	1.9	37
6	2.0	38	2.9	73	4.3	100	4.6	88
10	2.0	38	3.3	83	4.3	100	5.2	100
24	2.1	40	3.3	83	4.3	100	5.2	100

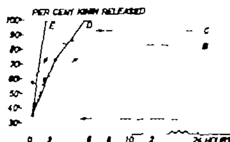


Fig. 1 *Significance of acetone plasma contact period for activation of kallikrein and inactivation of kallikrein inhibitor*

Plasma substrate was diluted with mixtures of acetone and saline to a final dilution of ½ (v/v). The mixtures were incubated at 37° for varying periods of time, acetone was evaporated, and saline added to a substrate dilution of ½ (v/v) (time 0). The mixtures were then further incubated and aliquots for assay were withdrawn at intervals. Kinin released was calculated as μg bradykinin/ml plasma and given as per cent of the maximum amount determined simultaneously by the acetone activation method (BRISEID, DYRUD & RERVIK 1967).

12 % acetone – – Contact period 10 minutes (A), 1 hour (B), 1½ hours (C).
16 % acetone ———— Contact period 10 minutes (D), 1 hour (E).

For further details see table 1 and text.

present to such an extent that maximum release is obtained The release activity was considerably less in the experiment in which 20 / acetone was used. Similar results were obtained with other substrate preparations

Fig. 1 shows for 12 / and 16 / (v/v) acetone how contact-time influenced the kinin release. With 12 / acetone, maximum kinin release was not obtainable after acetone evaporation, even if the acetone incubation period was prolonged to 2½ hours. On the other hand, with 16 / (v/v) acetone a contact period of 10 minutes sufficed for maximum release.

The absence of release of kinin at low acetone concentrations (table 1 2 / (v/v) acetone) might reflect the absence of activation of the releasing system It might, however also indicate the inactivation of activated kallikrein by inhibitors effective at such low acetone concentrations. To find out whether activation did in fact take place, experiments were carried out in which plasma substrate specimens were incubated with acetone, 2 / or 4 / (v/v), the acetone evaporated after 1 hour's contact period, and the kinin release subsequently determined with 10 / (v/v)

Table 5

Plasma kallikrein activity in plasma substrate incubated ith 2 % of acetone

I. Plasma substrate diluted with mixture of cetone nd saline to substrate dilution of ½ (/v) and an acetone concentratio of 2 % (/v) was incubated t 37 for 1 hour (Test 1), acetone was evaporated (½ hour), saline dded t riginal volume and the mixture was further incubated for 1 hou (Test 2).
Then
   A. The mixtur was further incubated
   B. An equal volume of plasma substrate nd saline was added t substrate dilution f ½ (/v) nd the mixture was further incubated.
   C. A mixture of acetone nd saline was added to substrate dilutio of ½ (v/v) and an acetone concentration of 10 % (/v) nd the mixture was further incubated (kininogen determination method of Briseid, Dyrud & Rinvik (1967).

II. Plasma substrate diluted with saline (½ /v) was incubated t 37 for ½ hours (Test 2)
F rther treatment as for I C.
Aliquots for assay were withdraw 4, 7 and 24 hours after point of time for Test 2

Kinin released	I						II	
	A		B		C			
	µg	%	µg	%	µg		µg	%
Test 1	0.5	12	0.5	12	0.5	12		–
Test	0.3	7	0.3	7	0.3	7	0.2	5
4 hours incub.	0.7	16	1.0	23	0.9	1	1 4	33
7 hours incub.	0.7	16	0.9	21	1.5	35	2.6	60
4 hours incub.			–	–	–	–	4.3	100

acetone. The results of a typical experiment are shown in table 5 A comparison of columns I C and II demonstrates a significant reduction in the rate of kinin release from the major kininogen fraction (BRISEID DYRUD & ARNTZEN 1968) caused by preincubation with 2 / (v/v) acetone this indicates a considerable activation and subsequent inhibition of factors in the release system. A comparison of columns I A and I B shows that after evaporation of the acetone, an enzyme fraction is active and capable of releasing kinin from the minor kininogen fraction (BRISEID DYRUD & ARNTZEN 1968) of fresh substrate added. That this fraction did not include active kallikrein, but possibly active Hageman factor was demonstrated in an experiment in which the addition of fresh substrate was replaced by the addition of fresh substrate heated at 60° for 1 hour in order to destroy prekallikrein. No release of kinin in the heat treated substrate was then observed. A control experiment with substrate heated to 60° and plasma kallikrein prepared by activation with 16 7 / (v/v) acetone for 17 hours (BRISEID DYRUD & ARNTZEN 1968) showed normal release of kinin corresponding to the minor kininogen fraction.

Table 6

Significance of plasma dilution for the activation of kallikrei and inacti ation of kallikrein inhibitors caused b acetone

Plasma substrate diluted with
I Acetone.
II. Mixtures of acetone and saline to final substrate dilution of ½ (/v)
III Mixtures f acetone nd saline t final substrate dilutio of ⅓ (/).
The mixtures wer incubated t 37 for 1 hour acetone was evaporated and saline added to a substrate diluti n of ⅓ (v/v) Then the mixtures were further incubated nd aliquots for assay were withdrawn at various times. Different batches of plasma substrate were used for the two acetone concentrations.
For further details see tables 1 and 3 and text.

Acetone conc. % (/)	12						16					
Kinin released after time in hours	I		II		III		I		II		III	
	µg	%	µg	%	µg	%	µg	%	µg	%	µg	%
1¾	–		–	–			2.0	47	1.3	30	1.3	30
3	2.0	41	1 6	33	1 5	31	3 4	79	[illegible].6	60	2.6	60
6	2.5	51	1.8	37	1.6	33	4.3	100	4.3	100	4 3	100
10	2.5	51	1.8	37	1.6	33						
24	2.8	57	2.2	45	2.2	45	4.3	100	4.3	100	4.3	100

Significance of the plasma dilution for the activation of kallikrein and inactivation of kallikrein inhibitors caused by acetone

Table 6 shows how the plasma dilution used in an one hour acetone contact period influenced the rate and extent of kinin release observed subsequent to acetone evaporation As previously demonstrated (table 4) contact with 12 / (v/v) acetone for one hour did not completely destroy the kallikrein inhibitors. The fact that the release of kinin at this acetone concentration came to a stop approximately after the same incubation periods (table 6) suggests that the extent of inhibitor inactivation is only insignificantly influenced by the degree of plasma dilution.

The increase in kallikrein activation with increasing plasma concentration suggested by the results in table 3 is also seen from the data shown in table 6 for both 12 / and 16 / (v/v) acetone.

Table 7

Determination of activities of kallikrein preparations obtained by incubating citrated plasma with different amounts of acetone for different periods of time

Incubations were carried out t oom temperature (22 ± 2°). The kininase activity was then eliminated by incubation with EDTA 2Na, 4 mg/ml citrated plasma, for 30 minutes at 37 The same batch of pooled citrated plasma was used for the preparation of the different kallikrein preparations examined. The same batch of plasma kininogen substrat was used in all experiments.
For further details see text and BRISEID, DYRUD & ARNTZEN (1968).

Experiment	Acetone: ml added/ml citrated plasma	Acetone: % (/v)	Incub. period hours	µl kallikrein prep./ml plasma substrate causing 50 % kinin release	Relative activity Test/Stand %
1 Test	0.10	9 1	17	>1000	<4
Standard	0.20	16 7	17	35	
2 Test	0.15	13.0	17	40	83
Standard	0.20	16.7	17	33	
3 Test	0.30	23 1	17	33	76
Standard	0.20	16.7	17	25	
4 Test	0.20	16.7	5	39	77
Standard	0.20	16.7	17	30	
5 Test	0.20	16.7	10	18	100
Standard	0 20	16.7	17	18	
6 Test	0.20	16.7	25	18	100
Standard	0.20	16.7	17	18	

Activities of kallikrein preparations obtained with different acetone concentrations and different incubation periods

Table 7 shows that the addition of 0.10 ml acetone per ml citrated plasma (9.1 % (v/v) in the incubate) was not sufficient to give an active kallikrein preparation after an incubation period of 17 hours at room temperature. Additions of 0.15 and 0.30 ml of acetone (13.0 and 23.1 % (v/v) in the incubates) yielded enzyme preparations which were almost equiactive, but clearly less active than the preparation obtained with the standard acetone concentration chosen for the assay procedure (BRISEID, DYRUD & ARNTZEN 1968), 0.20 ml per ml citrated plasma (16.7 % (v/v) in the incubate). Table 7 furthermore shows that the yield of kinin-releasing activity was the same for incubation periods of 10, 17 and 25 hours when 16.7 % acetone was used, and not much lower (77 %) if incubation was stopped after 5 hours.

Discussion

Acetone simultaneously activates prekallikrein, inhibits active kallikrein and inhibits inhibitors of kallikrein in human plasma. The rate and extent of kinin release caused by acetone accordingly depends on the balance between these effects, and determinations of released kinin allow no safe conclusions as to the separate effects. However, when the results of experiments in which acetone was continuously present were estimated together with the results of experiments in which the acetone was evaporated after varying periods of time, some information was obtained about the different effects of acetone.

Acetone concentration and activation of prekallikrein

The experiments suggest that even the lowest acetone concentration tested, 2 % (v/v), would activate some of the prekallikrein. Such an activation, followed by an inactivation by inhibitors, seems to be the most probable explanation of the fact that the usual rate of release of kinin caused by 10 % acetone was significantly reduced by preincubation of the plasma substrate with the lower acetone concentration (table 5). It should be mentioned that similar reductions in the rate of release of kinin were also noticed on preincubation with ellagic acid or with plasma kallikrein (unpublished experiments).

Acetone concentration and inactivation of kallikrein inhibitors

The lack of release of kinin at low acetone concentrations (2 % (v/v), table 1) together with the observation that some of the prekallikrein was

activated under such conditions (see above), is evidence that the kallikrein inhibitors are rather resistant to acetone inhibition First at about 10 / (v/v) of acetone a marked *inhibition* of kallikrein inhibitors was obtained, an inhibition which became *irreversible* with increasing acetone concentration and increasing contact time (tables 1 4 and 7 fig. 1). The acetone effect seemed to rise sharply from 12 to 16 / (v/v). At the first mentioned concentration a contact period of 2½ hours was not sufficient to destroy the inhibitors to such an extent as to cause a full release of kinin after removal of the acetone, while contact for 10 minutes with 16 / (v/v) acetone resulted in a maximum release.

Acetone concentration and inhibition of kallikrein

Preliminary experiments had shown that the activity of plasma kallikrein relative to the activity of its inhibitors was highest at an acetone concentration of about 10 / (v/v) This was demonstrated by registering the kinin releases in plasma substrate specimens brought about by a fixed amount of activated plasma kallikrein in the presence of varying concentrations of acetone (10 minutes incubation) The fact that kallikrein was less active at acetone concentrations lower or higher than 10 / (v/v) might indicate that the reversible inhibition of its inhibitors was complete at that concentration of acetone. Even if kallikrein was increasingly depressed by acetone concentrations over 10 / (v/v) a sharp rise in acetone induced kallikrein inhibition was first observed with 20 / (v/v) of acetone (table 1). The results shown in table 2 demonstrate that this inhibition was largely reversible.

Summary

The effect of different acetone concentrations on the release of kinin in human citrated plasma stabilized with EDTA was examined. By comparing the results of experiments carried out with acetone continuously present with the results of experiments in which acetone was evaporated after different incubation periods, information was obtained on the significance of the acetone concentration for the activation of kinin-releasing enzymes, the inhibition of such enzymes, and the inhibition of their inhibitors.

While some activation of kinin-releasing enzymes took place at the lowest acetone concentration tested, 2 / (v/v), about 10 / (v/v) of acetone was required for effective inhibition of the kallikrein inhibitors. The absence of release of kinin at low acetone concentrations (2 / v/v) re

flected the activation of prekallikrein and the subsequent inactivation of the kallikrein by inhibitors present.

Evidence was provided that the very low kinin release values observed on incubation of plasma substrate with 20 / (v/v) acetone were primarily due to a reversible, acetone-dependent inhibition of factors in the releasing system.

As a basis for a method for determining plasma prekallikrein, conditions were established for the preparation by acetone of a plasma kallikrein preparation of maximum activity An acetone concentration of about 16 7 / (v/v) in citrated plasma gave the highest yield of activity The yield was the same for incubation periods of 10 17 and 25 hours, and not much lower when incubation was stopped after 5 hours.

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Epiphyseal Growth Zones in Cortisone-Treated Rabbits

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Histological studies of rabbit epiphyseal growth zones have provided an explanation for the changes induced by cortisone (SISSONS & HADFIELD 1955, STOREY 1957, HULTH & WESTERBORN 1963). Biochemical studies of cortisone treated animals have mainly been carried out on cartilage and bone tissue other than epiphyseal cartilage and metaphyseal bone.

The most important structural changes are narrowing of the cartilage plate and progressive resorption of the metaphyseal trabeculae, eventually leading to bony closure of the growth zone.

Some supplementary quantitative data on epiphyseal cartilage and metaphyseal spongiosa are given here.

Method

Weaning rabbits, about two weeks of age, were used. In order to obtain sufficient tissue material for the analyses, two identical experiments were carried out. The first experiment (tables 1–4) included 7 controls and 9 experimental animals (3 batches of 5, 5 and 6 animals). The second experiment (table 5, figs. 1 & 2) comprised 6 controls and 6 experimental animals (2 batches of 5 and 7 animals). Initial mean body weights of control and experimental animals were 250 ± 10 g and 253 ± 7 g, respectively, in the first experiment and in the second experiment the animals weighed 200 ± 19 g and 212 ± 18 g. Animals and respective mothers were caged in groups with free access to food and water, the diet containing 0.9 % Ca and 0.6 % P.

The experimental animals received an injection of 1 mg cortisone acetate (Cortison CIBA ®, 10 mg/ml) per 100 g body weight daily over four days. The injections were given into the posterior femur muscles, alternating between right and left sides. The control animals received a corresponding volume of physiological saline.

Carrier-free radiosulphate was diluted with 0.04 % Na_2SO_4 to a final concentration of 1 mcu/ml. On the day of the last cortisone or saline injection, a dose of approximately 1 µcl per g of body weight was injected intraperitoneally into all the animals.

With no further treatment, the animals were sacrificed two days later by an intraperitoneal injection of ml 6 % nembutal ® (mebumal) and the bones of all four extremities removed. Under a dissection microscope, the epiphyseal cartilages and the corresponding metaphyseal bone plates were removed from the proximal humerus and tibia, as well as from the distal radius, the ulna and the femur (BERNTSEN 1967 a).

Following dissection, the wet weights were determined and the tissue dried to constant weight over P_2O_5 in an Edwards tissue drier at a pressure of less than 0.5 mm Hg. The samples of cartilage were crushed to a powder in small glass grinder and the bone samples were powdered in a Wiley mill after defatting (BERNTSEN 1967 a).

The cartilage and bone tissues were then analysed for hydroxyproline, hexosamine and uronic acid. The uptake of $^{35}SO_4$ was measured and the content of sodium, potassium and chloride determined. The analytical techniques have previously been published in detail (BERNTSEN 1966, BERNTSEN 1967 b &).

Hydroxyproline analyses were made according to the modified (MARTIN & AXELROD 1953) NEUMAN & LOGAN (1950) procedure.

Hexosamine analyses were based on modifications (BOAS 1953, KIRK & DYRBYE 1956, DYRBYE 1959) of the ELSON & MORGAN (1933) procedure.

Uronic acid. Isolation of mucopolysaccharide was done according to BOLLET *et al.* (1958), applying a scaled-down version (MARCKMANN 1965 b) for the bone samples. Uronic acid was determined by the carbazole (DISCHE 1947) as well as by the orcinol method (BROWN 1946).

Radioactivity measurements were carried out as described by MOLTKE (1957) and MARCKMANN (1965 a). After addition of carrier-sulphate and wet ashing with fuming HNO_3, the samples were precipitated with $BaCl_2$, filtered, dried and counted at infinite thickness with a G M tube, to a statistical counting error of less than 3 %. Count rates were corrected for background, physical isotope decay, variations in the amounts of tissue and variations in the animals body weight.

Electrolytes. Sodium and potassium were determined by flame photometry (LANGGÅRD 1965) and chloride by electrometric titration with $AgNO_3$, in acetic acid (COTLOVE 1963, LANGGÅRD 1965).

The results were statistically evaluated according to Student's "t"-test.

Results

Grossly the cortisone-treated animals were indiscernible from the controls throughout both experiments. In the first experiment, the mean body weight curves followed each other closely during the first four days, while the experimental curve presented a deflection towards lower values on the last two days. On day 6 the mean body weight of the controls was 363 ± 15 g, as against 325 ± 12 g for the experimental animals ($P < 0.05$). In the second experiment, the weight curves ran a parallel course, the final body weights for the control and experimental animals being 276 ± 26 g and 287 ± 22 g.

In both experimental groups, no gross skeletal lesions were evident at autopsy. However, during dissection, the epiphyseal cartilage appeared definitely drier and the tissue mass was obviously reduced. No macroscopic alterations were observed in the metaphysis.

Table 1

Wet weights, dry weights and water of epiphyseal cartilage and metaphyseal bone of control and cortisone-treated rabbits.

Experimental animals were injected intramuscularly with 1 mg cortisone acetate (0.1 ml Cortison CIBA ®) per 100 g body weight daily for 4 days. Control animals received a corresponding volume of physiological saline. All animals were sacrificed 2 days after the last injection.
Figures are mean values ± e.m.
Figures in brackets indicate number of animals.
Experimental values differ significantly from co trol values at the 5% ( ) or 0.1% (*) levels of probability (*) indicates significant change.

		Wet weight mg	Dry weight mg	Water % of wet wt.
Cartilage	Controls (7)	374 ± 16	66.5 ± 2.9	82.3 ± 0.1
	Experim (9)	145 ± 8	30.5 ± 1.4	78.8 ± 0 6
Bone	Controls (7)	541 ± 23	192 ± 9	64.4 ± 0.4
	Experim. (9)	459 ± 18	180 ± 8	60.7 ± 0.3

In table 1 the average amounts of cartilage and bone tissue recovered, and the water content are given. A reduction in the wet as well as the dry weights of cartilage was observed. In bone a decrease in the wet weight was noted, whereas the dry weight decrease was not significant. The water content decreased in both tissues. Identical changes were observed in the second experiment except that the decrease in wet weight of bone was not significant.

Table 2

Content of hydroxyproline, hexosamine nd uronic acid epiphyseal cartilage of control and cortisone-treated rabbits.

Animals are identical with those of table 1
Figures are mean values ± s.e m.
Experimental hydroxyproline differs significantly from the control value t the 1 / ( ) level of probability (*) indicates no significant cha ge.

µg/mg dry tissue	Hydroxy-proline	Hexos-amine	Uronic acid Carbazole	Uronic acid Orcinol
Controls (7)	14 3 ± 0.3	58.9 ± 1.0	93 7 ± 2.2	56.5 ± 1 7
Experim. (9)	16.1 ± 0.5	60.1 ± 1 7	93 4 ± 1 6	56.5 ± 1 1

Table 3

Content of hydroxyproline, hexosamine and uronic acid in metaphyseal bone of control and cortisone-treated rabbits.

Animals are identical with those of table 1
UA Hex = Hexosamine calculated a 1 1 molar basis with uronic acid = Carbazole value × 179 194
Figures are mean values ± s.e.m.
One experimental orcinol sample was lost during analysis.
Experimental values differ significantly from control values at the 1 % (*) or 0.1 % ( ) levels of probability (*) indicates no significant change.

μg/mg dry defatted tissue	Hydroxy-proline	Hexosamine			Uronic acid	
		Total	UA Hex	Excess	Carbazole	Orcinol
Controls (7)	11.0 ± 0.3	4 67 ± 0.08	1 16 ± 0.02	3.51 ± 0.07	1.26 ± 0.03	0.79 ± 0.04
Experim (9)	11 3 ± 0.4	5.35 ± 0.05	1 65 ± 0 09	3 71 ± 0.10	1 79 ± 0 09	1.06 ± 0.06 (8)

Table 4

Uptake of $^{35}SO_4$ in epiphyseal cartilage and metaphyseal bone of control and cortisone-treated rabbits, in relation to dry tissue weight and to hexosamine content.

Animals are identical with those of table 1. On the day of the last injection, all animals received one intraperitoneal injection of approximately 1 µci of $^{35}SO_4$ per g of body weight. UA Hex cf table 3.

Radioactivity ratios denote the proportions cartilage to bone count rates (cpm/mg : cpm/mg)

Figures are mean values ± s.e.m.

Experimental values differ significantly from control values at the 0.1% ( ) level of probability. (*) indicates no significant change.

Cartilage		Bone		Radioactivity ratios	
Controls (7)	Experim. (9)	Controls (7)	Experim. (9)	Controls (7)	Experim. (9)
counts/min./mg dry tissue		counts/min./10 mg dry defatted tissue		Cartilage/Bone	
2257 ± 143	405 ± 25	831 ± 58	252 ± 13	27.3 ± 0.9	16.1 ± 0.6
counts/min./µg hexosamine		counts/min./µg UA Hex		Cartilage/Bone	
38.8 ± 2.6	6.7 ± 0.4	71.3 ± 4.1	15.6 ± 1.2	0.55 ± 0.02	0.46 ± 0.04

The biochemical determinations are shown in tables 2 (cartilage) and 3 (bone). The hydroxyproline concentration increased in cartilage but remained at the control level in bone. The hexosamine and uronic acid concentrations were unchanged in cartilage, whereas increased concentrations were observed in bone. The hexosamine increase in bone was presumably related to the acid mucopolysaccharide.

The radioactivity measurements (table 4) demonstrated an inhibition of the $^{35}SO_4$-uptake in cartilage and bone. The lower experimental radioactivity ratios indicate that this inhibition was more marked in cartilage than in bone.

Table 5 gives the electrolyte determinations and also the dry tissue weights and the water content of the tissues in this experiment. Significant electrolyte changes consisted of a decrease in cartilage and bone chloride and in bone potassium.

In fig. 1 and 2 water and electrolytes were recalculated to [illegible] to the actual amounts of dry tissue, and the following [illegible]

Table 5

Sodium, potassium, chloride, dry tissue weights and water content of epiphyseal cartilage and metaphyseal bone of control nd cortisone-treated rabbits.

Dry tissue = dried cart lage and dried defatted bone.
Animals were treated as indicated in legends to table 1
Figures are mean values ± s.e.m.
Figures in brackets indicate umber of animals.
E perimental alues differ significantly from control values t the 5% ( ), 1% (*) or 0.1% ( ) levels f probability (*) indicates no significant cha ge.

		meq/100 g dry tissue			Dry tissue mg	Water g/100 g dry tissue
		Na	K	Cl		
Cartilage	Controls (6)	76 ± 2.8	49 ± 1 6	30 ± 0.9	55 ± 3	411 ± 10
	Experim. (6)	69 ± 0.9	44 ± 1 6	24 ± 1 1	30 ± 2	351 ± 10
Bone	Controls (6)	24 ± 0.7	18 ± 0.4	12 ± 0.3	151 ± 19	221 ± 9
	Experim (6)	23 ± 0.3	16 ± 0.3	10 ± 0.2	136 ± 11	193 ± 8



Fig. 1 Dry tissue (DT), water and electrolytes of epiphyseal cartilage control (left) and cortisone-treated rabbits (right). Animals are identical with those of table 5. Water is given as g, dry tissue as mg and electrolytes as meq. Cross-hatched bars represent meq Na and K per 100 g dry tissue. EC = extracellular. IC = intracellular

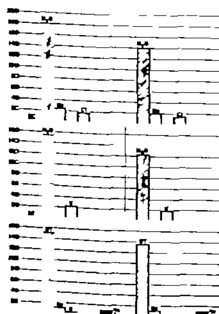


Fig. 2. Dry (defatted) tissue (DT), water and electrolytes of metaphyseal bone of control (left) and cortisone-treated rabbits (right). Animals are identical with these of table 5. Units as in fig. 1

predominant distributions were then assumed Chloride = 100 meq/l (extracellular), sodium = 140 meq/l (extracellular) and potassium = 150 meq/l (intracellular) The extracellular water was derived from the chloride value, i.e. total minus extracellular water being intracellular water From the amounts of water it was possible to calculate the respective amounts of meq sodium and potassium. "Surplus" sodium and potassium were considered osmotically inactive (USSING *et al* 1960) and related to the dry tissue.

Discussion

The dissimilar weight curves of the experimental groups remain unexplained, as both experiments were carried out under identical conditions. Inhibition of weight gain is to be expected during treatment with corticosteroids (WELLS & KENDALL 1940), even if treatment is of short duration and the dosage of the steroid moderate (SMITH & ALLISON 1965). The importance of dietary Ca/P balance to body weight as well as to osteoporosis has been emphasized (STOREY 1960 & 1961) but the diet appeared adequate in this respect.

In epiphyseal cartilage, the recovery of dry tissue and the water content are in agreement with the macroscopic findings, and apparently independent of the change in body weight. In metaphyseal bone, the decrease in the amount of wet tissue seems to be largely due to the decreased water content. If estimated by the amount of dry tissue, no essential bone resorption occurred.

An estimation of the changes in the water content of cartilage and bone was attempted by means of the electrolyte determinations (fig. 1 and 2) It is evident that the amounts of extra and intracellular water represent approximations. A true expression of the extracellular water was not obtained by a calculation based on chloride, nor were the different subgroups of extracellular water (LANOGÅRD 1967) and the hydration water of bone crystals (NEUMAN & NEUMAN 1957) taken into consideration

However the results suggest that the nature of the water decrease was different in the two tissues While the decrease in cartilage was confined to the extracellular water both extra and intracellular water was affected in bone. It also appeared that the sum of osmotically inactive sodium and potassium per unit dry weight was the same in the control and experimental tissues.

The effect of corticosteroids on collagen and mucopolysaccharides in costal cartilage, in embryonic tissue cultures in metaphyseal and in compact bone has previously been investigated The results of hydroxyproline (SOBEL & MARMORSTON 1954 URIST & DEUTSCH 1960 KOWALEWSKI 1962 REYNOLDS 1966), hexosamine (SOBEL & MARMORSTON 1954

URIST & DEUTSCH 1960 KOWALEWSKI 1962 KAPLAN & FISCHER 1964 SCHRYVER 1965 REYNOLDS 1966) and uronic acid determinations (WHITEHOUSE & LASH 1961 KAPLAN & FISCHER 1964) are inconsistent, possibly because of the considerable variations in the experimental conditions Inhibition of collagen synthesis (DAUGHADAY & MARIZ 1962 SMITH & ALLISON 1965) and of the uptake of $^{35}SO_4$ (BOSTRÖM & ODEBLAD 1953 KOWALEWSKI 1958 LOTMAR 1960 DENKO & BERGENSTAL 1961 WHITEHOUSE & LASH 1961 DAUGHADAY & MARIZ 1962 HULTH & WESTERBORN 1963) has been demonstrated.

When comparing the concentrations of hydroxyproline, hexosamine and uronic acid with the total amount of dry tissue, decreased concentrations were to be expected in epiphyseal cartilage if the reduced amount of dry tissue was due to a reduction only of collagen and mucopolysaccharide. However the hydroxyproline increased while the hexosamine and uronic acid were unchanged. The analytical results lead to the assumption that a loss of other compounds may also be responsible for the low amount of dry matter since this would affect the reference (mg dry tissue) and thus 1) mask an acid mucopolysaccharide decrease so that the concentration remains unchanged, and 2) result in a false increase in hydroxyproline.

The indication of a relatively smaller inhibition of $^{35}SO_4$-uptake in the experimental metaphyses should be evaluated in connection with the increase in metaphyseal hexosamine and uronic acid and furthermore with regard to the possible origin of metaphyseal acid mucopolysaccharides from the epiphyseal cartilage (DZIEWIATKOWSKI *et al.* 1957 BERNTSEN 1966 BERNTSEN 1967b). Taking previous results into account (BERNTSEN 1968), an acceleration of the "transfer" of cartilaginous mucopolysaccharides into the metaphysis may well occur

Consequently the increase in the mucopolysaccharide observed in the experimental metaphyses probably represents an actual increase. However the amount of dry tissue was not significantly changed, and there was even a small non-significant decrease. Provided the metabolism of other tissue components was undisturbed, a decrease in the hydroxyproline concentration irrespective of an inhibition of collagen synthesis, would be expected, but the concentration remained unchanged. As in cartilage, the results are therefore suggestive of a loss of unidentified tissue compounds, although the nature of these may not necessarily be the same in the two tissues.

Summary

The effect of short-term cortisone treatment on the epiphyseal growth zones of young rabbits is described A decreased water concentration was

observed both in cartilage and in bone, and the average dry tissue weight of cartilage decreased Based on electrolyte determinations, it was tentatively concluded that the water decrease was related to the extracellular water in cartilage and to the extra as well as intracellular water in bone. The sulphation of cartilage and bone was markedly inhibited. Cartilage to bone radioactivity ratios as well as acid mucopolysaccharide concentrations in bone, point to an acceleration of the "transfer" of labelled compounds from cartilage to bone. Evaluation of hydroxyproline and mucopolysaccharide concentrations in connection with the dry tissue weights suggests that a loss of undetermined compounds may occur in cartilage and bone

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Adrenergic Nerve Function in the Anaesthetized Rat after Treatment with α Methyldopa

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The ability of α-methyldopa (L-α-methyl-3 4-dihydroxy-phenylalanine, α MD) to lower arterial blood pressure in animals and man is generally thought to depend on an interference with the sympathetic nervous system (reviews by SOURKES 1965 MUSCHOLL 1966 HOLTZ & PALM 1966 STONE & PORTER 1966 & 1967) However the localisation of the action of the drug within this system has not yet been established The decrease in blood pressure may be brought about by an effect of α MD on the central nervous system, leading to a reduced sympathetic activity or to an effect on the peripheral adrenergic nerves, or both The relative importance of these components is poorly understood. While there are several indications of functional impairment in the central nervous system, opinions differ as to the effect of the drug on the peripheral sympathetic system. A number of reports indicate that α MD may cause a moderate blockade of this system while other investigators have obtained normal responses. (For references, see reviews mentioned above and Discussion of this paper).

A sympathetic blockade of importance for the antihypertensive effect of α MD should be easily demonstrable at the time of maximal fall in blood pressure following the administration of the drug. In a previous study (HENNING 1967) it was found that the administration of a single dose of α-MD lowered the blood pressure of conscious normotensive rats. A maximal decrease was observed 3–6 hours after the injection. In the present investigation we have carried out some tests of adrenergic nerve function on anaesthetized rats during this period following the administration of the same dose of α-MD The tests included lower eyelid contraction to cervical sympathetic stimulation and eyelid and blood pressure responses to injections of a ganglionic stimulant drug.

4-(m-chlorophenylcarbamoyloxy)-2 butynyltrimethyl ammoniumchloride (McN A 343) and also of carbachol after atropine. In addition the eyelid and blood pressure responses to injections of eserine, tyramine and noradrenaline were studied. The results suggest that adrenergic function was but little affected and the responses to tyramine and noradrenaline were about the same in the control rats and in rats treated with α MD

Methods

Male Sprague-Dawley rats weighing 200–300 g which had been bilaterally adreno-demedullated 2–3 weeks bef re the experiments (FARRIS & GRIFFITH 1949) were anaesthetized with urethane 1.2 g/kg s.c. A tracheal cannula was inserted and both vagi were cut. Drugs were injected into the right jugular vein via a polyethylene catheter Arterial blood pressure was recorded from the left carotid artery with a Statham Model P23Dc pressure transducer and a Grass Polygraph. For recordings of the contractions of the lower eyelid to nerve stimulation we used the technique described by OBIANWU (1967). The right cervical ympathetic trunk was carefully isolated and stimulated preganglionically by a Grass Model S4G stimulator Supramaximal stimulation voltage and impulse duration (5V 1 msec.) were used. The steady state contractions of the lower eyelid were recorded semi-isometrically by a Grass Model FT-03 force displacement transducer The initial tension was set to 0.50 g.

L-α-methyldopa was administered intraperitoneally in dose of 400 mg/kg, dissolved in 3–4 ml 0.9% N Cl and the experiments were performed 3–6 hours after the injection. Usually treated and a control rat were studied simultaneously Care was taken to prevent hypothermia. The following drugs were used in the experiments 4-(m-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride (McN A 343) carbachol chloride, tyramine hydrochloride, atropine sulphate, eserine salicylat and ( )-noradrenaline-o-bitartrate. The doses are given under Results and always refer t the salts. All drugs were dissolved in 0.9 % NaCl and injected intravenously except in the case of atropine, the total volume was always djusted to 0.25 ml. The blood pressure responses of the drugs are expressed as the maximal response after intravenous injection.

Results

1 *Contraction of lower eyelid to nerve stimulation*

In control rats, appreciable contractions were observed after stimulation of the cervical sympathetic trunk with 0 8 imp /sec. Increasing the frequency gave an almost linear response of the eyelid up to about 25 imp./sec. (fig. 1A)

Three to six hours after the administration of α MD 400 mg/kg the responses to all frequencies seemed to be slightly lower than in the control animals (fig. 1A) However the difference between the two groups was not statistically significant (Student s t-test for various frequencies or regression coefficients for the two dose-response curves)

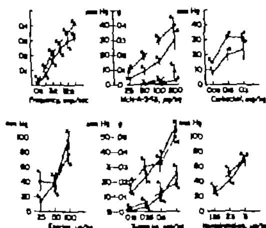


Fig. 1 Adrenergic function in adreno-demedullated rats under urethane anaesthesia. Open symbols indicate control animals and solid symbols indicate rats given L-α-methyldopa 400 mg/kg 3–6 hours before the experiment. Contractions of lower eyelid were recorded as increases in tension (g)

A. Contraction of lower eyelid to preganglionic stimulation of the cervical sympathetic trunk (3 V 1 msec.).
B. Increase in blood pressure (circles) and contraction of lower eyelid (triangles) to intravenous injections of McN A 343.
C. Increase in blood pressure to intravenous injections of carbachol chloride after atropine sulphate 10 mg/kg i.v. ten minutes previously
D. Increase in blood pressure to intravenous injections of eserine salicylate.
E. Increase in blood pressure (circles) and contraction of lower eyelid (triangles) to intravenous injections of tyramine-HCl.
F. Increase in blood pressure to intravenous injections of ( )-noradrenaline bitartrate.

2. *Basal blood pressure*

The basal mean arterial blood pressure in rats used for control experiments was 103 mm Hg (s.e.m. = 4.6 n = 19). Animals which had received α MD 400 mg/kg intraperitoneally about 3 hours previously seemed to have a lower blood pressure (91 mm Hg, s.e.m. = 4.3 n = 14) but the difference was not statistically significant ($p < 0.25$). Most previous investigations indicate that α-MD has no hypotensive effect after systemic administration to anaesthetized animals (for references, see reviews mentioned above). This is in contrast to the conscious rat in which a significant lowering of mean arterial pressure was observed 3 hours after α-MD 400 mg/kg intraperitoneally (HENNING 1967). In this study the basal blood pressure was 116 mm Hg (s.e.m. = 2.1 n = 14) which is significantly different from that observed in the present investigation ($p < 0.05$). The level attained after α MD in conscious rats (after

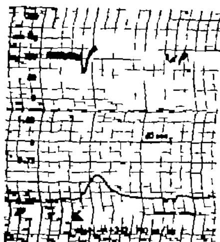


Fig. 2. Blood pressure (upper curve) and lower eyelid (lower curve) responses to an intravenous injection of McN A 343 100 μg/kg. Adrenodemedullated rat, ♂ 270 g, urethane 1.2 g/kg s.c.

3 hours 96 mm Hg, s.e m. = 3.2, n = 13) was not different from that in anaesthetized rats ($p < 0.50$).

3 *Blood pressure and lower eyelid responses to drugs*

a) *McN A 343* 4-(m-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride, McN A 343 is considered to be a selective ganglion stimulant for the peripheral sympathetic system in various species. It does not seem to have any significant action on the parasympathetic ganglia (Roszkowski 1961 Smith 1966) After intravenous injection of the drug a transient fall in blood pressure occurs which is followed by a pressor effect. The above-mentioned investigators have shown that the increase in blood pressure is reduced or abolished following surgical sympathetic ablation or administration of α-adrenergic receptor blocking agents. The pressor response is absent in animals pretreated with bretylium or reserpine (Roszkowski 1961).

In our experiments intravenous injections of McN A 343 to adrenodemedullated rats produced a small initial decrease in blood pressure which was followed by an increase of 40–80 seconds duration (fig. 2). The dose-response curve was straight up to about 200 μg/kg (fig. 1 B). Larger doses were found to cause respiratory stimulation in addition to the effect on blood pressure and were therefore not included in the study At the higher doses a small contraction of the inferior eyelid was sometimes observed (fig. 1 B). No tachyphylaxis was observed in one experiment

when eight injections of 400 μg/kg were given in rapid succession In rats pretreated with reserpine 10 mg/kg 24 hours previously (two experiments) or epsilon amino caproic acid (EACA) 2 g/kg 6 hours previously (two experiments), injections of McN A 343 did not produce any appreciable increases in blood pressure or eyelid contractions.

Three to six hours after α-MD 400 mg/kg there seemed to be a reduction in the blood pressure responses to McN A 343 at all dose ranges (fig. 1 B). However the regression coefficients for the two dose response curves were not significantly different from each other ($p < 0.50$) The contraction of the eyelid also appeared to be reduced after α-MD but statistical significance was not obtained.

b) *carbachol after atropine*. Intravenous injections of carbachol chloride to adrenodemedullated rats pretreated with atropine sulphate (10 mg/kg i.v ten minutes before) gave short-lasting increases in blood pressure. No effect was usually observed on the lower eyelid. The dose- response curve appeared to be rather steep since carbachol 0.08 mg/kg gave a small effect while 0.15 and 0.30 mg/kg seemed to give a maximal response (fig. 1 C). In rats given α MD before the experiments the responses to the two lower doses of carbachol were lower than in control animals for 0.15 mg/kg the difference was significant ($p < 0.01$).

c) *eserine salicylate* In the urethane-anaesthetized rat intravenous injections of eserine produces an increase in blood pressure (DIRNHUBER & CULLUMBINE 1955 VARAGIĆ 1955).

Fig. 1 D represents the results of the present study Rather marked individual variations in the sensitivity of the rats to eserine were noted and the rise in blood pressure tended to be long-lasting, particularly after the largest doses (10–30 minutes). Animals pretreated with α-MD responded less to the smallest dose of eserine, but, owing to the large individual variations, the difference was not significant. At higher doses of eserine no difference was observed

d) *tyramine hydrochloride* Intravenous injections of tyramine produced increases in blood pressure and contractions of the inferior eyelid (fig. 1 E) Pretreatment with α-MD had no effect on the blood pressure response but the contractions of the eyelid seemed to be larger after tyramine 1 mg/kg. However statistical significance was not obtained.

e) ( )-*noradrenaline-bitartrate*. Fig. 1 F shows the dose response curves for the increase in blood pressure after various doses of noradrenaline (NA) in untreated rats and after α MD 400 mg/kg. Usually no effect was observed on the lower eyelid in these experiments. As seen in the figure, no alteration in sensitivity to NA was seen after α MD

Discussion

In the present investigation the response of the rat lower eyelid to electrical stimulation of the cervical sympathetic was but little impaired 3–6 hours after a large dose of α-MD i.e. at the time of maximal decrease in blood pressure, when the same dose of the drug was given to conscious rats (HENNING 1967). At the same interval the increase in blood pressure after ganglionic stimulation by injections of McN A 343 or carbachol after atropine may have been slightly depressed in the rats pretreated with α-MD.

As stated in the introduction, previous studies on the function of the peripheral sympathetic nerves after α-MD have frequently yielded variable and even conflicting results. These will be discussed in more detail below. If relevant to the mechanism of the hypotensive action of α-MD, studies of this kind should be conducted at a time when an effect on blood pressure of the drug is apparent. It appears that this occurs for only a relatively short interval after the administration of single doses, usually about 12 hours, and in long-term studies the blood pressure returns to control levels in about the same time (for references, see reviews mentioned in the introduction). During this time no relaxation of the nictitating membranes or alteration in the circulatory response to tilting was seen in the conscious dog (GOLDBERG *et al.* 1960). These investigators also found that the positive chronotropic and inotropic responses to preganglionic stimulation of the cardiac sympathetic nerves in anaesthetized dogs were not blocked by previous administration of α-MD, but the time of observation was not stated. DAY & RAND (1964) found no decrease in the blood pressure response to bilateral carotid occlusion 110 minutes after a large intravenous dose of α-MD to anaesthetized cats. This finding was confirmed by DAVIES (1966) who, in addition, found that pressor responses to stimulation of the splanchnic nerves were the same before and after intravenous α-MD. HAEFELY *et al.* (1967a & b) observed normal blood pressure responses to graded doses of the ganglion stimulant dimethylphenylpiperazinium (DMPP) and normal contractions of the isolated perfused spleen four hours after the last of several previous doses of α-MD had been given. KĄDZIELAWA (1967) found normal responses of the nictitating membranes in anaesthetized cats after α-MD in doses up to 500 mg/kg given intravenously during the experiments. Responses to sympathetic stimulation in the isolated rabbit ileum and guinea-pig vas deferens were not impaired, in animals pretreated with several doses of α-MD, the last of which was given six hours before the experiment was started (DAY & RAND 1964).

However some observations seem to indicate that α-MD may indeed suppress peripheral sympathetic function at short intervals In their previously-mentioned study DAY & RAND (1964) obtained a clear cut reduction in the contractions of the cat nictitating membrane to pre ganglionic sympathetic stimulation 2 and 5 hours after a single dose of α-MD The effect was most marked at low stimulation frequencies. After acute administration of α MD to dogs, the chronotropic responses to stimulation of the accelerans nerves were markedly reduced at all fre quencies tested (SUGARMAN *et al* 1965) Further HAEFELY *et al* (1967a & b) studied the peripheral adrenergic reactivity in cats at various intervals, after pretreatment with α MD in different dosage schedules and found an effect on sympathetic transmission only when the last dose was given shortly before the experiment. Responses of the nictitating membranes to preganglionic stimulation were moderately reduced at all frequencies and the heart rate increases to injections of DMPP were less than in control animals. However as quoted above, certain sympathetic responses were not affected by α MD in this study ROBSON (1967) examined the blood pressure responses to graded doses of McN A 343 in the anaesthet ized rat one and six hours after a single dose of α MD and obtained a depression of the effect at all doses tested.

A large number of studies have been performed at longer intervals after the administration of α MD (usually 16–24 hours). In the majority of these experiments, the sympathetic nerves to the spleen, heart and nictitating membranes appear to have functioned normally (STONE *et al* 1962 MUSCHOLL & MAÎTRE 1963 STONE & STAVORSKI 1963 VARMA & BENFEY 1963 SCHAEPDRYVER *et al* 1963 HAEFELY *et al* 1966 & 1967a & b KADZIELAWA 1967) However DAY & RAND (1964) stated that the cat nictitating membrane contractions to nerve stimulation were reduced in similar experiments and SCHAEPDRYVER *et al* (1963) found that the vasoconstriction after nerve stimulation in the dog s hind leg was abolished 24 hours after a large dose of α MD

It seems almost impossible to draw any definite conclusions about these conflicting results, which have been obtained under widely different conditions. Thus, several animal species are involved either conscious or under various types of anaesthesia A large number of different tests of adrenergic function have been performed at varying intervals after administration of α MD Most likely there are species differences and the sensitivity of the sympathetic nerves to the action of the drug may vary in different tissues. In particular the type of pretreatment with α MD seems to influence the results. As is evident from the survey of previous work given above, most investigators seem to find a normal sympathetic

transmission when the drug is given several hours before the test. If on the other hand, the interval is relatively short, a moderate adrenergic blockade is sometimes observed. This effect then coincides with the period when the blood pressure is lowered.

It should however be stressed that such a blockade, if it occurs, is always considerably less pronounced than that observed after adrenergic neurone blocking drugs or reserpine. In experiments similar to those in the present study epsilon amino caproic acid (EACA) was found to inhibit almost completely the contractions of the inferior eyelid to electrical stimulation and the blood pressure increase to carbachol after atropine (ANDÉN *et al.* 1968). When given to conscious rats in these doses, EACA produced a hypotensive effect of the same magnitude as that obtained after α-MD in the dose used in the present investigation. Reserpine, which blocks the transmission from postganglionic sympathetic neurones (CARLSSON *et al.* 1957, MUSCHOLL & VOGT 1958, ANDÉN *et al.* 1964), lowered the mean arterial blood pressure in conscious rats to about the same extent as α-MD and EACA (HENNING unpublished observations). Thus, these two drugs have an effect on the blood pressure similar to that of α-MD though differing greatly from this drug in their action on the peripheral adrenergic nerves. This seems to indicate that the impairment of peripheral sympathetic function by α-MD is not of sufficient magnitude to account exclusively for the hypotensive effect of this drug in the rat.

In our experiments, the increases in blood pressure to graded doses of eserine were not significantly altered by pretreatment with α-MD. This finding contrasts with the results of BRUNNER *et al.* (1967) who observed a significant reduction of the response to a large dose of eserine. However in this case α-MD was given daily in large doses for 11 days before the experiments. If as proposed by VARAGIĆ and coworkers (LEJIĆ & VARAGIĆ 1961, VARAGIĆ & VOJDOVIĆ 1962), eserine increases blood pressure mainly by a central activation of the sympathetic system, one would expect α-MD to reduce the response. Several other examples of functional impairment in the central nervous system after α-MD are known (for references, see HENNING & VAN ZWIETEN 1968). It appears, however that several factors may be involved in the mechanism of the hypertensive action of eserine. Thus, LALANNE *et al.* (1966) in an extensive investigation also obtained evidence for a peripheral component, probably localized in the autonomic ganglia. Considering the feeble action of α-MD on various peripheral adrenergic mechanisms, the lack of effect on the response to eserine is compatible with the view of LALANNE *et al.* (1966), that this drug indeed has a predominantly peripheral effect.

No significant alteration of the blood pressure and lower eyelid responses to tyramine was observed in the present investigation. BENTLEY &

VARMA (1963) also found normal cardiovascular responses to tyramine, using spinal cats pretreated with moderately large doses of α-MD At higher doses, however they found a significant depression of these responses. This observation is not substantiated by subsequent studies in several species after various types of pretreatment with α MD over a wide dose-range, all of which have failed to demonstrate a significant effect of α-MD on tyramine responses (KRONEBERG & STOEPEL 1963 DAY & RAND 1964 ROSS 1965 HAEFELY *et al.* 1966 & 1967a & b) The investigations referred to so far have involved acute experiments or pre treatment for a few days. An enhanced pressor response to tyramine after α-MD has been reported following long-term administration of the drug in humans (PETTINGER *et al.* 1963 MCCURDY *et al.* 1964) These observa tions might be explained by the development of a supersensitivity to NA released by tyramine, but the last-mentioned investigators found no significant increase in the response to infusion of NA. On the other hand, an increased sensitivity to this amine has been demonstrated in animals receiving α-MD for several days before the experiment (SUGARMAN *et al* 1965 HAEFELY *et al* 1966 & 1967b) Following short-term pretreatment with α-MD no supersensitivity to NA develops, as is evident from our own results as well as those of BRUNNER *et al.* (1966) and HAEFELY *et al* (1967b). A complete understanding of the interaction of α MD with the responses to tyramine is probably not possible until a more detailed analysis of the effects of the two drugs on the adrenergic neurone is available.

The results of the present investigation as well as the survey of previous work given above, indicate that the responses of the peripheral adrenergic nerves may be moderately reduced at the time of maximal effect on blood pressure after α-MD The impairment is considerably less marked than that observed after adrenergic neurone blocking drugs and reserpine. However these drugs lower the blood pressure to about the same extent as α MD suggesting that the peripheral effects of the latter drug are not solely responsible for its hypotensive action. In a previous communication (HENNING & VAN ZWIETEN 1968) evidence has been obtained that the blood pressure lowering effect of α-MD may be at least in part, of central nervous origin. The exact mechanism of this effect requires further investigation.

An explanation for the hypotensive action of α MD was put forward by DAY & RAND (1964). According to their theory treatment with α MD leads to the formation of α methylnoradrenaline which is released from the sympathetic nerves in place of NA, thus acting as a substitute or "false" transmitter Since the receptor activity of α-methylnoradrenaline is thought to be less than that of NA the effectiveness of sympathetic

impulses is diminished, leading to a reduction in blood pressure. Taken in this restricted sense, the false transmitter concept is difficult to reconcile with the weak action of α MD on peripheral adrenergic nerves. Further there is no clearcut time correlation between the NA depletion in peripheral organs and the decrease in blood pressure (HENNING 1967). On the other hand, mechanisms of this kind may operate in central nervous neurones. Assuming that this is the case, the theory of false transmission may still be relevant to the hypotensive effect of α MD

Summary

Various tests of peripheral adrenergic function were applied in urethane anaesthetized adreno-demedullated rats 3–6 hours after α methyldopa (α MD) 400 mg/kg i p This interval has previously been shown to correspond to the period of maximal decrease in blood pressure in conscious animals.

No significant difference between the initial blood pressure levels was obtained. In rats treated with α MD there was a small and insignificant decrease in the response of the lower eyelid to electrical stimulation of the cervical sympathetic trunk. Stimulation of the sympathetic ganglia by injections of McN A 343 produced increases in blood pressure which were insignificantly decreased in pretreated rats. The increase in blood pressure after carbachol 0 15 mg/kg in atropinized rats was significantly lower after α MD No significant reduction was observed in the blood pressure responses to graded doses of eserine, tyramine and noradrenaline. Lower eyelid contractions to tyramine were likewise unaltered

The sympathetic blockade after α MD seems to be considerably less marked than that observed after adrenergic neurone blocking drugs such as epsilon amino caproic acid (EACA) or reserpine. These drugs lower blood pressure in conscious animals to about the same extent as α-MD which suggests that the moderate impairment of peripheral adrenergic mechanisms by the latter drug is not sufficient to account *per se* for the hypotensive action of this drug.

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Distribution of Adrenergic Receptors in the Urinary Bladder of Cats, Rabbits and Guinea-Pigs

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Electrical stimulation of the hypogastric nerve in cats elicits a biphasic mechanical response of the urinary bladder consisting of an initial contraction followed by an inhibition of the spontaneous activity and tone this results in closure of the bladder outlet and relaxation of the corpus.

This biphasic effect may be due to stimulation of two types of nerve fibres in the hypogastric nerve. Different nerve fibres could not, however be found by electrical stimulation at the threshold intensities of the hypogastric nerve (EDVARDSEN 1967) That the effect is caused by the release of two different transmitters is also unlikely since neither atropine nor ganglion blocking agents abolished the biphasic response (EDGE 1955 EDVARDSEN 1967). Cholinergic fibres, therefore, do not appear to be involved in the response.

The most likely explanation is that the smooth muscles of the cat urinary bladder contain excitatory as well as inhibitory adrenergic receptors. The response to adrenergic agents may be due to a biphasic response in one muscle fibre or to excitatory and inhibitory responses respectively of fibres located in different parts or in different muscle layers of the bladder

The aim of the present investigation has been to study the distribution of adrenergic excitatory and inhibitory receptors in the urinary bladder

The whole bladder *in vivo* has not been found suitable for the clarification of this problem and the results of intra-arterial and intravenous injection of noradrenaline have been conflicting. In the cat a bladder contraction (EDGE 1955 SIGG & SIGG 1964) or a relaxation (SIGG & SIGG 1964) has been recorded following an intra-arterial injection and a relaxation after intravenous administration (EDGE 1955).

In the present investigation the response to adrenergic drugs of small pieces of fresh muscular tissue isolated from different parts and layers of the urinary bladder has been studied as well as tissue "denervated" by cold storage.

Materials and Methods

I vivo stimulation of the hypogastric nerves in 6 anesthetized cats (chloralose-urethane 40 and 80 mg/kg respectively) was performed after laparotomy The hypogastric nerves were sectioned nd isolated i one tubular stimulation electrode, the stim l being delivered through st mulus isolation unit. The bladder responses nder isotonic conditions ere recorded on smoked drum fter insertion of an urethral catheter

In the *vitro* experiments, the urinary bladders from 4 cats, 15 rabbits and 6 guinea-pigs of either sex were used. The guinea pigs were killed by a blow on the neck and the rabbits by air embolism the animals were bled nd the bladder then removed. In the cats the bladder was removed under ether anaesthesia. For each experiment, one piece of muscular tissue of about 5 mm in length was dissected from the bladder base (between the urethra and th ureters) The piece was removed either by a circular or by a longitudinal incision in the bladder Another piece of tissue was taken from the detrusor muscle (the bladder d me) either transverse or parallel to the longitudinal axis of the bladder The preparations were ot taken from the same part of the bladder each time, but at different sites so as t obtain pieces distributed all over the bladder Usually 6 or 8 preparations were removed from each bladder

Recording technique

The mecha ical activity of both muscles was recorded in the same orga bath with mechano-electrical transducer valves (RCA 5734) connected to two channel Hewlett Packard recorder (7702A). The modified Krebs solution, which was kept at 36 contained (mM) N 136.9 K 5 9 Ca^{2} 2.5 Mg^{2} 1.2, HCO_3 15 5 H_2PO 1.2, Cl^- 133.6 and glucose 11.5 equilibrated with a gas mixture of 5% CO_2 and 95 O_2.

Cold storage After the first preparation had been removed, the bladder was stored in Krebs solution at 4 Pieces ere examined after storage for 24 48 nd 72 hours. An equ libration period of bout hours was allowed before the experiment was started.

Adrenergic and adrenergic blocking agent Adrenaline bitartrate, noradrenaline bitartrate and isoprenaline were added t the organ bath to produce concentrations in the bathing solution from 10^{-9} to 10^{-3} g/ml. Propranolol (Inderal ®, ICI) was used as β-blocking agent, phenoxybenzamine and phentolamine (Regitin ®, Ciba) as blockers of the adrenergic receptor

Results

In vivo stimulation of hypogastric nerves

The bladder response to hypogastric nerve stimulation was biphasic consisting of a rapid contraction followed by relaxation As can be seen from fig. 1 the contraction was capable of expelling about half of the bladder content when the bladder outlet was kept open by means of a

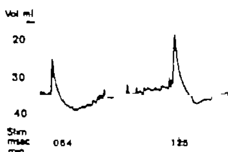


Fig. 1 The recording of the changes in volume of the cat urinary bladder response to hypogastric nerve stimulation.

urethral catheter (isotonic conditions) Moreover the maximum relaxation was obtained after cessation of stimulation. The response varied in proportion to the duration of the stimulus.

In vitro experiments

(a) *Spontaneous rhythmic activity* Fig. 2 shows that the muscle strip from the bladder base and the bladder dome show a marked difference both with regard to the amplitude as well as to the frequency of spontaneous contractions. The rhythmic contractions of the detrusor muscle strips are marked, whereas those of the strips from the bladder base are insignificant or absent. Such observations were constantly obtained when simultaneous recording from the two parts of the bladder musculature was made in the same organ bath. The direction in which the muscle strips were excised from the bladder wall did not alter the pattern of the rhythmic activity

It thus appears, that in spite of the lack of anatomical differences in the muscle cells of the detrusor and of the bladder base (BRO-RASMUSSEN *et al* 1965), the different spontaneous contractions indicate different functional characteristics of the smooth muscle from these parts of the bladder

(b) *Response to adrenergic drugs* The typical response patterns of the two muscle strips on addition of adrenaline, 10^{-7} g/ml, are shown in fig. 2. Adrenaline caused relaxation of the strip from the detrusor muscle and contraction of the strip from the bladder base, indicating the presence of inhibitory receptors in the former and excitatory receptors in the latter Furthermore, the excitatory response had a shorter latency than the inhibitory response. Noradrenaline yielded essentially the same results as adrenaline, but the responses were quantitatively weaker Isoprenaline in this case had no effect on the strip from the bladder base whereas the activity of the detrusor strip was markedly inhibited on adding this drug.

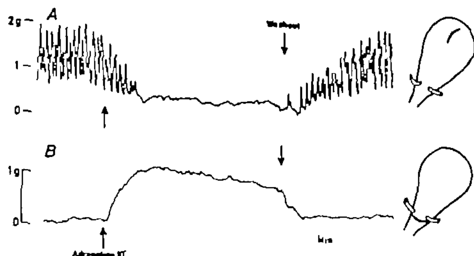


Fig. 2. The response to adrenaline of muscle strips from the dome (A) and the base (B) of the rabbit urinary bladder Isometric recording. Fresh preparations.

Fig. 3 The inhibitory response of a strip from the cat bladder detrusor muscle to nor adrenaline (A) adrenaline (B) and isoprenaline (C) is blocked by propranolol. The responses to noradrenaline and adrenaline are reversed in the propranolol treated muscles. These excitatory responses are blocked by phenoxybenzamine. 4 hrs. cold storage.

(c) *Effect of blocking agents* The inhibition induced in the muscle strip from the *detrusor muscle* by the addition of isoprenaline, was completely prevented by propranolol (10^{-6} g/ml). Moreover the effect of adrenaline and noradrenaline was reversed by propranolol, the inhibition being substituted by an increased tension of the muscle strip (fig. 3). This contraction of the detrusor muscle strips due to adrenaline and noradrenaline after blockade of the inhibitory receptors with propranolol was abolished when phenoxybenzamine (10^{-7} g/ml) was also added to the solution (fig. 2). No excitatory response was obtained after the inhibitory response to isoprenaline (10^{-7} g/ml) had been blocked by propranolol. These results thus indicate that both excitatory α and inhibitory β-receptors are present in the detrusor muscle, but that the α response is overcome by the inhibitory action of β-receptors, and thus normally escapes observation.

The response to adrenaline of the muscle strips from the *bladder base* as reported above, was a contraction. After adding propranolol (10^{-6} g/ml) to the solution this response was unaltered or even increased (fig. 4B). On the other hand, additional administration of phentolamine (10^{-5} g/ml) or phenoxybenzamine (10^{-7} g/ml) to the bathing solution abolished the response completely (fig. 4C) When phentolamine was given before propranolol, an inhibitory response was occasionally

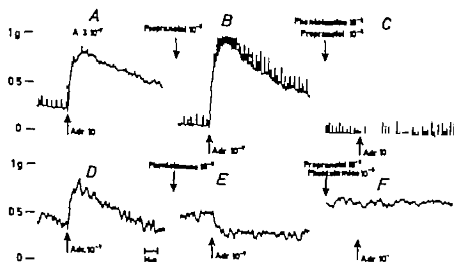


Fig. 4 The excitatory response to adrenaline in fresh preparation from the rabbit bladder base (A) is increased fter propranolol (B) and blocked by phentolamine (C). If the phentolamine is given first, an inhibition ppears in preparation taken from the same region after 24 hrs. cold storage (D E). The latter is blocked by propranolol (F).

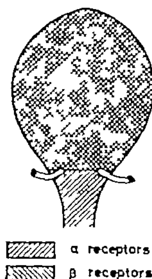


Fig. 5 The distribution of drenergic excitatory (α) and inhibitory (β) receptors in the urinary bladder of the cat and rabbit.

observed, and this latter response could again be blocked by propranolol (fig. 4E & F)

These results suggest that both α and β- receptors are also present at the bladder base. However in this region the α response predominates, so that the β-response, which was observed less constantly might have been due to the presence of detrusor muscle fibres in the preparations investigated The results of *in vivo* stimulation of the hypogastric nerves do not support the suggestion of inhibitory responses in the bladder base (SIGG & SIGG 1964 GJONE 1965 EDVARDSEN 1967).

(d) *Distribution of receptors* As shown by the diagram (fig. 5) the primary α and β- responses were obtained from the areas of the bladder which were separated by the interureteral crest. The inhibitory response of the detrusor strips showed no significant difference whether the strips contained circular or longitudinal muscle fibres. On the other hand, the clearest α responses of the strips taken from the bladder base were obtained from circular preparations and from Bells muscle.

(e) *Species differences* No species differences were observed in muscle strips from the cat and rabbit bladder On two occasions muscle strips from the base of the human bladder were investigated and in these preparations the α-response was conspicuous. On the other hand, detrusor muscle strips from the guinea-pig showed no addition of catecholamines to the soluti α in strips from the bladder base. Ti do exist

regarding the bladder response to adrenergic stimulation (McLean & Burnstock 1967).

(f) *Cold storage* The preparations were kept at 4 in order to "denervate the tissue (Vogt 1943). After 24 hours both the inhibitory and excitatory response was more marked than in the fresh preparations. A qualitively similar response was also obtained after storage for 72 hours. These results indicate that the observed responses of muscle strips from various parts of the bladder to catecholamines are due to receptor activity

Discussion

The present experiments indicate that in the urinary bladder adrenergic β-receptors are present in the detrusor muscle of the dome where they produce a marked inhibitory response. In addition α-receptors are present at the bladder base where they elicit a contraction in response to adrenergic stimulation. These receptors are, however also present in the bladder dome.

The significance of the α-receptors in the bladder base is probably to ensure bladder continence, since stimulation of the hypogastric nerves is followed by contraction of the bladder outlet (Learmonth 1931 Edvardsen 1967). Furthermore, a sympathetically induced contraction in sexual excitation prevents retrograde ejaculation into the bladder (Barrington 1915 Sacks 1966). On the other hand, the significance of these receptors in the detrusor muscle is not clear According to the present experiments the adrenergic contraction is initiated before the relaxation and this contraction becomes marked and sustained when the β-receptors are blocked This observation is in agreement with experimental observations *in vivo* (Ingersoll & Jones 1958 Edvardsen 1967) and may explain the different results obtained after intra-arterial administration of noradrenaline reported by Edge (1955) and Sigg & Sigg (1964)

Synergistic effects of concomitant pelvic and hypogastric nerve stimulation have not been observed by *in vivo* recording (Edvardsen 1967) On the other hand, recordings of hypogastric nerve efferent activity showed that although the efferent activity increased in proportion to the filling of the bladder consistent with increased bladder inhibition there was a sharp rise in the activity when spontaneous micturition occured (Edvardsen unpublished). It is unlikely that this increased efferent activity indicates inhibition, and hence it is tempting to assume that the inhibitory activity in the detrusor produced by the hypogastric nerve by some unknown mechanism, can be blocked or converted to an excitatory effect.

When the inhibitory receptor of the detrusor muscle is activated, it

relaxes the bladder and the bladder is thereby allowed to retain more fluid without a proportional increase in the intravesical pressure. In the present experiments this receptor was constantly found in the feline and in the rabbit but not in the guinea-pig bladder Apparently this receptor is developed to a varying degree in different species. Preliminary experiments seem to indicate that the β-receptor is active in man.

According to the present experiments the old concept of a reciprocal innervation of the detrusor muscle and the internal vesical sphincter by the sympathetic nerves (LEARMONTH 1931) seems to be true Although the internal sphincter is discarded as an anatomical structure (CLEGG 1957 BRO-RASMUSSEN *et al* 1965), the bladder base apparently constitutes a functional sphincter The closing effect of the latter in addition to the detrusor relaxation in response to the adrenergic transmitter provides a most effective combination for ensuring bladder continence.

Summary

Electrical stimulation of the hypogastric nerve *in vivo* elicits a biphasic response, i.e. an initial contraction followed by inhibition of the spontaneous rhythmic activity and tone. To clarify this biphasic effect the adrenergic receptors in small pieces of muscular tissue taken from sites distributed all over the bladder were studied.

Muscle strips taken from the urinary *detrusor muscle* of cats and rabbits exhibited vigourous rhythmic activity whereas these contractions were small or absent in muscle taken from the bladder base. The detrusor muscle strips were found to respond to adrenaline, noradrenaline and isoprenaline with an inhibition which was blocked by propranolol, indicating that β-receptors are involved However these muscles also possess α-receptors since the responses to adrenaline and noradrenaline are reversed by propranolol These excitatory responses in turn are again blocked by phenoxybenzamine

In muscles from the *bladder base* adrenaline and noradrenaline elicited contractions indicating activation of α-receptors. These responses are sometimes increased after propranolol. When the excitatory responses are blocked by phentolamine or phenoxybenzamine, inhibitory responses occur indicating the presence of β-receptors. Cold storage did not abolish the responses.

The excitatory response has a shorter latency than the inhibitory one. The initial contraction after hypogastric nerve stimulation is therefore most likely due to a contraction of the whole bladder

Acknowledgements

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Relationship between some Metabolic Effects of Nicotinic Acid and Catecholamines in the Rabbit

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The lowering effect of nicotinic acid on blood cholesterol has attracted great interest (review ALTSCHUL 1964) Nicotinic acid also depresses the level of other lipid fractions of the blood, such as unesterified fatty acids (FFA) triglycerides and phospholipids (CARLSON & ORÖ 1962 CARLSON & NYE 1966) The lipid mobilizing effects of catecholamines and ACTH are also blocked by nicotinic acid (CARLSON & ORÖ 1962 CARLSON & BALLY 1965) The FFA lowering effect of nicotinic acid is sometimes followed by an FFA-increasing action (CARLSON & ORO 1962 PEREIA 1967) Nicotinic acid influences other metabolic processes as well as the lipid metabolism. The blood sugar may be increased or depressed (MIRSKY *et al* 1957 ALTSCHUL 1964) The glucose tolerance was found to be decreased in patients receiving large doses of nicotinic acid and in a number of cases a moderate hyperglycaemia was demonstrated (BERGE & MOLNAR 1964). In the rat and mouse however large doses had a hypoglycaemic action (CARLSON & NYE 1966 AMMON & ESTLER 1967) This same effect was demonstrated after small doses of nicotinic acid in man (HARTHON LUNDHOLM & SVEDMYR 1968) In the fasted rat, nicotinic acid stimulated the gluconeogenesis and urea production (TROUT BITTER & LOCKEY 1967). In man chronic administration of nicotinic acid increased the basal metabolism (ALTSCHUL & HOFFER 1958) wheras acute administration had no effect (SVEDMYR 1966a)

The calorigenic effect of noradrenaline and adrenaline was reduced in man by nicotinic acid (HAVEL *et al* 1964 STEINBERG *et al* 1964 SVEDMYR 1966a) and this reduction was attributed to an inhibition of the FFA mobilizing effect of the catecholamines. In the rabbit the calorigenic

action of adrenaline has mainly been ascribed to an increase in the production and metabolism of lactate (LUNDHOLM 1949 SVEDMYR 1966b LUNDHOLM, MOHME-LUNDHOLM & SVEDMYR 1966). According to RUDMAN *et al* (1965) the catecholamines have no FFA-mobilizing effect *in vivo* in the rabbit. SVEDMYR (1966b), however found that in this species adrenaline induced a distinct, though transient, increase in the plasma FFA content, while the FFA-elevating effect of noradrenaline was of longer duration. The short duration of this metabolic action of adrenaline may explain why it was missed by RUDMAN *et al* (1965).

In view of these manifold metabolic actions of nicotinic acid we considered it of interest to study some of them further in the rabbit. We investigated 1 the effect of nicotinic acid itself on the carbohydrate, FFA and the basal metabolism, 2. the question of whether an adrenergic β-blocking agent influenced these effects of nicotinic acid and 3 the way in which this compound influenced the metabolic actions of catecholamines

Method

The experiments were performed on unan esthetized rabbits which had been fasted for 16–18 hours. A cannula was introduced into the marginal vein f one ear for the infusion of nicotinic acid and/or catecholamines, while another cannula in the central artery of the other ear was used for the withdrawal of blood samples for analyses. The oxygen consumption and also the concentrations of FFA, glucose and lactat in the blood were determined according to methods described previously (SVEDMYR 1966b). I ll experiments the basal oxygen consumption was first recorded over 10-min. periods for at least 90 min. A basal blood sample was then taken for determination of the FFA, lactate and glucose concentrations. I the first series of experiments nicotinic acid, as the N salt, was nfused for 10 min. in dose of 5 15 or 100 mg/kg body weight. Blood samples were taken 25 70 and 150 min. fter the start of the infusion In the second series of experiments, -4-(2-isopropylamino-1-hydroxy-ethyl)methane-sulphonan lide HCL (MJ 1999) was i fused in dose f 15 mg/kg 10 m n. prior to an infusion of nicotinic acid in the same dose In third series of experiments either L-adrenaline or L-noradrenaline was infused dose of 0.5 µg/kg/min. f 60 m Blood samples were taken 15 60 and 150 mi after the start of the infusion. In order t obtai sufficiently large material for statistical analysis, the results f some experiments reported previously (LUNDHOLM & SVEDMYR 1964 SVEDMYR 1966b) have been included ith those of the adrenaline experiments. The catecholamine experiments included infusion of combination of nicotinic acid and adrenaline or noradrenaline. I the experiments with adrenaline, nicotinic acid in a dose f 5 mg/kg was first infused for 10 min followed immediately by an infusion of adrenaline for 60 min. In dose of 0.5 µg/kg/min Blood samples ere taken 15, 60 and 150 min. after the start of the adrenaline infusion I the experiments with noradrenaline, nicotinic acid in a dose of 15 mg/kg was first infused, followed by noradrenaline dose f 0.5 µg/kg/min.

I all experiments the oxygen consumption was recorded over period of 150 min. starti g t the beginning of the infusion.

Table 1

I fluence of adrenaline (A 0.5 μg/kg/min.) and nicotinic acid (nic.ac. 5 mg/kg) and nic.ac. in combination with A on oxygen consumption FFA level of plasma and glucose and lactate concentrations of blood. Means ± S.E.M n = no. of tests. Significance levels $P < 0.05$ = $P < 0.02$ = $P < 0.001$ =

	1 A	2 Nic.ac. (n = 5)	3 Nic.ac. + A (n = 6)	Difference 3–(1 + 2)
Oxygen consumption ml/kg min.				
Basal values	9.05 ± 0.47 (n = 25)	9.31 ± 0.39	7.94 ± 0.25	–
Change after: 0–60 min.	1.09 ± 0.11	0.17 ± 0.21	0.30 ± 0.11	−0.96 ± 0.26
60–120 min.	1.38 ± 0.15	0.71 ± 0.22	2.27 ± 0.28	0.18 ± 0.38
FFA meq/l				
Basal values	1.17 ± 0.09 (= 8)	0.83 ± 0.19	0.89 ± 0.08	–
Change after 15 min.	0.52 ± 0.08	−0.29 ± 0.10	−0.13 ± 0.06	−0.36 ± 0.14
60	− 0.01 ± 0.07	−0.07 ± 0.05	0.02 ± 0.05	0.10 ± 0.11
150	−0.23 ± 0.09	−0.14 ± 0.04	−0.18 ± 0.07	0.19 ± 0.12
Glucose mg/100 ml				
Basal values	84 ± 3 (n = 8)	90 ± 5	99 ± 6	–
Change after 15 min.	117 ± 11	4 ± 12	76 ± 9	−45 ± 19
60 min.	261 ± 33	4 ± 6	226 ± 35	−39 ± 48
150 min.	55 ± 13	1 ± 5	100 ± 20	44 ± 25
Lactate mg/100 ml				
Basal values	6.7 ± 0.7 (n = 25)	6.2 ± 0.5	8.5 ± 1.2	–
Change after 60 min.	44.7 ± 2.3	0.5 ± 1.0	56.4 ± 2.8	11.2 ± 3.8

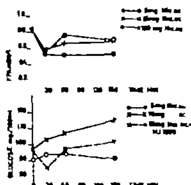


Fig. 1 Influence of nicotinic acid in different doses per/kg and MJ 1999 (15 mg/kg) on the FFA concentration of plasma and glucose concentration of blood.

Results

The results are shown in figs. 1 and 2 and tables 1 2 and 3

Nicotinic acid. In a dose of 5 mg/kg, nicotinic acid induced an increase in the oxygen consumption of about 8 / of the basal value 70–130 min. after the start of the infusion (table 1) The plasma FFA concentration was reduced, but there were no significant effects on the glucose and lactate concentrations of the blood (fig. 1) With a higher dose (15 mg/kg), the reduction in FFA became more prolonged. In no case was there any indication of a secondary increase in the FFA level A weak hyperglycaemic effect, which was most marked after 150 min. was noted after the infusion of 15 mg/kg (fig. 1). A still higher dose of nicotinic acid (100 mg/kg n = 7) had a more prolonged FFA-reducing effect (fig. 1) After this dose, the oxygen consumption during 0–120 min. increased by 0 68 ± 0.21 ml/kg/min. ($P < 0.02$). This dose of nicotinic acid also had a slight hyperglycaemic effect.

Nicotinic acid and adrenergic β-receptor blocking agent There is evidence that stimulation of the sympathetic nervous system increases the FFA level of the plasma (HAVEL 1964). One possible explanation of the FFA lowering effect of nicotinic acid was that it blocked the sympathetic action. It seemed of value to determine whether the hyperglycaemic or calorigenic effect of nicotinic acid could be elicited by stimulation of adrenergic receptors. We therefore investigated the effect of an adrenergic β-blocking agent (MJ 1999 15 mg/kg) on the metabolic actions of nicotinic acid in a dose of 15 mg/kg. In another investigation we had ascertained that MJ 1999 almost completely blocked the metabolic actions of adrenaline (AABERG *et al.* 1968)

Table 2

Influence of nicotinic acid (nic.ac.) (15 mg/kg) MJ 1999 ot-4-(2 isopropylamine 1 hydro y-ethyl)methane-sulfona ilde HCL (15 mg/kg) ad nic.ac. in combination with MJ 1999 on oxygen consumption, plasm FFA nd blood glucose concentrations. Significance levels s in t ble 1

	1 Nic.ac. (n = 8)	MJ 1999 (n = 10)	3 MJ 1999 Nic.ac. (n = 6)	Difference 3 − (1 + 2
Oxygen consumption ml/kg/min.				
Basal values	7.7 ± 0.3	8.2 ± 0.5	7.9 ± 0.2	–
Change after 0–120 ml	0.52 0.15	0.42 ± 0._7	0.56 ± 0.10	−0.38 0.3
FFA meq/l				
Basal values	0.51 0.07	1.14 ± 0.20	0.52 ± 0.0	
Change after 25 min.	0.79 0.04	0.08 ± 0.08	−0.23 ± 0.03	−0.08 _ 0.10
70 min.	0.18 0.05	0.0_ 0.09	−0.18 ± 0.05	−0.02 ± 0.11
130 min.	0.18 0.06	−0.16 ± 0.11	0.16 ± 0.06	0.18 ± 0.15
Glucose mg/100 ml				
Basal values	97 6	95 ± 6	96 7	–
Change after 25 min.	6 5	0 ± 3	−10 ± 4	−16 ± 7
70 min.	10 ± 10	−4 ± 4	1 ± 4	−5 ± 11
130 min.	18 ± 5	4 ± 4	6 ± 4	−16 ± 7

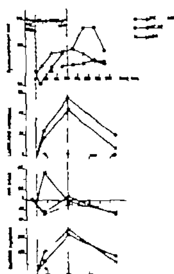


Fig. 2. Influence of nicotinic acid (5 mg/kg) adrenaline (0.5 μg/kg/min for 60 min.) and nicotinic acid in combination with adrenaline on oxygen consumption in per cent of the basal value and the lactate FFA and glucose concentrations of blood. Changes from basal values

MJ 1999 had no significant metabolic action of its own It blocked the hyperglycaemic effect of nicotinic acid (table 2 fig. 1). The FFA lowering and calorigenic actions of nicotinic acid were not influenced, however by adrenergic blockade

Catecholamines A detailed report has been made previously on the metabolic effects of adrenaline and noradrenaline in the rabbit (SVEDMYR 1966b). Adrenaline had a distinct calorigenic action and markedly increased the lactate and glucose contents of the plasma. Initially the FFA was clearly increased but this effect disappeared despite continuous adrenaline infusion, and was changed to an FFA-reducing action (table 1 fig. 2) The magnitude of the calorigenic effect of noradrenaline was about half that of adrenaline. Its FFA increasing effect was much more prolonged than that of adrenaline. The hyperglycaemic and, particularly the lactate-increasing effect of noradrenaline were weaker than those of adrenaline (table 3 fig. 3).

Nicotinic acid and catecholamines in combination. An attempt was made to determine whether nicotinic acid inhibited or potentiated the effects of the catecholamines. Tables 1 and 3 give the separate effects of the catecholamines (1) and nicotinic acid (2) substracted from their combined effect (3). If a summation occurred, the difference 3-(+ 2) would be zero

Table 3

Influence of noradrenaline (NA) (0.5 µg/kg/min.), nicotinic acid (15 mg/kg) (nic.ac.) and nicotinic acid in combination with NA on oxygen consumption, plasma, FFA concentration and glucose and lactate concentrations of blood. Significance levels as in table 1

	1 NA (n = 7)	2 Nic.ac. (n = 8)	3 Nic.ac. NA (n = 8)	Difference 3 − (1 + 2)
Oxygen consumption				
Basal value, ml/kg/min.	8.3 ± 0.3	7.7 ± 0.3	7.8 ± 0.3	
Mean increase over basal value 0–120 min. ml/kg/min.	0.61 ± 0.10	0.52 ± 0.15	0.66 ± 0.11	−0.47 ± 0.[illegible]
FFA meq/l				
Basal value	1.01 ± 0.10	0.85 ± 0.09	0.98 ± 0.14	–
Change after: 15 min.	0.57 ± 0.09	−0.27 ± 0.06	−0.37 ± 0.81	−0.66 ± 0.13
60 min.	0.51 ± 0.13	−0.19 ± 0.04	−0.08 ± 0.04	−0.40 ± 0.14
150 min.	−0.13 ± 0.07	−0.16 ± 0.05	−0.30 ± 0.09	−0.01 ± 0.13
Glucose, mg/100 ml				
Basal value	90 ± 3	97 ± 6	105 ± 5	–
Change after 15 min.	34 ± 6	6 ± 5	62 ± 4	[illegible] 8
60 min.	48 ± 4	10 ± 10	101 ± 12	43 ± 16
150 min.	13 ± 4	18 ± 5	16 ± 9	−15 ± 11
Lactic acid, mg/100 ml				
Basal value	6.2 ± 0.7	6.8 ± 0.8	6.9 ± 0.6	–
Change after: 15 min.	3.6 ± 0.7	1.1 ± 1.1	2.4 ± 0.8	−2.3 ± 1.5
60 min.	1.5 ± 0.9	0.5 ± 0.8	1.9 ± 0.5	−0.1 ± 1.3
150 min.	1.0 ± 0.9	1.4 ± 0.6	0.5 ± 0.6	−1.9 ± 1.3

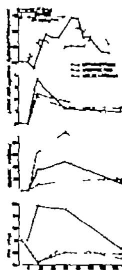


Fig. 3. Influence of noradrenaline (0.5 μg/kg/min.), nicotinic acid (15 mg/kg) and nicotinic acid + noradrenaline on oxygen consumption, FFA concentration in plasma and glucose and lactate concentrations of the blood. Changes from basal values.

If nicotinic acid had an inhibitory effect the difference would be negative, and the opposite effect would give a positive difference.

Nicotinic acid and adrenaline in combination (fig. 2) resulted in a reduction of the oxygen consumption to 91 ± 2.7 / (P < 0 02) of the basal value during the first 20 min. of the infusion. This reduction did not occur when adrenaline was given alone. During the 60 min. infusion of adrenaline the effect was significantly lower in the animals treated with nicotinic acid than in those which had not received nicotinic acid (table 1 fig. 2) During the following 60–120 min., on the other hand, the adrenaline effect following nicotinic acid was much greater When the difference 3-(1 + 2) was calculated for the whole period (0–120 min.) there was no significant decrease in the oxygen consumption (—0 35 ± 0.28 ml/kg/min.).

Nicotinic acid completely inhibited the FFA raising effect of adrenaline but the hyperlactacidaemic action was increased (table 1 fig. 2).

The fact that the calorigenic action of adrenaline was not blocked by nicotinic acid during the 60–120 min. period might have been due to disappearance of the nicotinic acid effect. In the tests with noradrenaline, nicotinic acid was therefore administered in a dose of 15 mg/kg.

The FFA-increasing action of noradrenaline was totally blocked by nicotinic acid. The hyperglycaemic effect was potentiated however (table 3 fig 3)

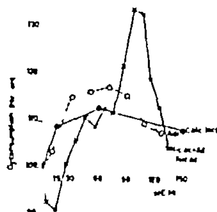


Fig 4 Calcul ted effect of adren l ne in combination with nicotinic acid (Nic.ac + Adr Nic. ac.) and the observed effect of adren line (Ad) on oxygen cons mption. Increase of oxygen consumption calculated from increased blood lactate in the Nic.ac. + Adr tests (Calc.lact.)

The calorigenic action of noradrenaline and nicotinic acid in combination was significantly less than the total effects of noradrenaline and nicotinic acid given alone (table 3) This indicated that nicotinic acid partly inhibited the calorigenic action of noradrenaline The inhibition was probable dependent on the blockade of the FFA mobilization

Discussion

Metabolic actions of nicotinic acid Our experiments on the rabbit showed that nicotinic acid reduced the FFA level in plasma had a slight hyperglycaemic effect and stimulated the basal oxygen consumption The hyperglycaemic effect of noradrenaline was potentiated whereas the calorigenic action was reduced As mentioned in the introduction, nicotinic acid has also other metabolic actions

A priori it is probable that some of the many metabolic actions of nicotinic acid are related to one other and that a few are direct effects and most of the others indirect effects. An interesting question is the nature of the primary action or actions of nicotinic acid and how this or these are related to its other metabolic actions.

CARLSON & ORÖ (1962) and CARLSON & NYE (1966) have suggested that the lowering effects of nicotinic acid on cholesterol, triglycerides and phospholipids are a concequence of the blockade of the FFA mobilization This may not be the whole truth however as nicotinic acid blocks

the FFA mobilizing effect of noradrenaline without reducing its cholesterol-increasing action (KWAM 1965)

It is probable, however that blockade of the FFA-mobilization is one of the primary actions of nicotinic acid. An understanding of this mechanism is therefore of importance. There is some evidence that the FFA lowering effect of nicotinic acid may depend on an inhibition of the action of some stimulating agent on FFA mobilization. Fasting is known to increase the FFA level of plasma. The effect of nicotinic acid on the FFA level was most pronounced in fasting animals (GARRATINI & BIZZI 1966) The basal FFA release from isolated fat pads of fed rats was not influenced by nicotinic acid whereas that from rats fasted for 48 hours, was reduced (CARLSON & BALLY 1965) On the other hand, nicotinic acid still reduced the plasma FFA level after adrenergic β-receptor blockade and also in adrenalectomized and hypophysectomized fasted rats (FARKAS *et al* 1964 PEREIRA 1967). The origin and nature of the suggested stimulating agent in fasting animals is therefore not clear

The FFA-mobilizing action of hormones such as the catecholamines, glucagon and peptid hormones of the hypophysis is probably mediated by a stimulation of adenyl cyclase and increased level of cyclic 3–5 AMP in the tissue (BUTCHER *et al* 1965) The lipolytic effect of these hormones and of cyclic AMP is associated with an increased activity of triglyceride lipase (RIZAK 1964 STEINBERG 1966) Nicotinic acid has been shown to inhibit the lipase activation produced by catecholamines (BJÖRNTORP 1966) In tests on brown fat tissue we found that nicotinic acid selectively blocked the FFA-mobilizing effect of noradrenaline and of added cyclic AMP Other actions such as the phosphorylase activating effect of noradrenaline which is also mediated by cyclic AMP was not blocked by nicotinic acid (BEVIZ *et al.* 1968). These results indicate that nicotinic acid inhibit the action of cyclic AMP on the FFA mobilization but not the formation of cyclic AMP

We therefor suggest that one primary action of nicotinic acid is to block the lipase activating effect of cyclic AMP

It is probable however that some of the more indirect metabolic effects of nicotinic acid are also related to a stimulation of the adenyl cyclase-cyclic AMP system The FFA rebound which may follow large doses of nicotinic acid was absent in hypophysectomized and adrenalectomized rats, and associated by an increased level of corticosterone (PEREIRA 1967) It is probable that nicotinic acid either directly or by a reflex action, liberates a hormone with an FFA-mobilizing action and which may act via cyclic 3–5 AMP

The hyperglycaemic effect of nicotinic acid was blocked by the adrenergic β-blocking agent, and nicotinic acid potentiated the hyperglycaemic

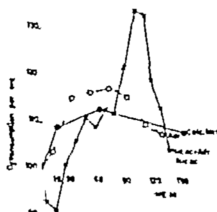


Fig. 4 Calculated effect of adrenaline in combination with nicotinic acid (Nic.ac. + Adr Nic. ac.) and the observed effect of adrenaline (Adr) on oxygen consumption. Increase of oxygen consumption calculated from increased blood lactate in the Nic.ac. + Adr test (Calc.lact.)

The calorigenic action of noradrenaline and nicotinic acid in combination was significantly less than the total effects of noradrenaline and nicotinic acid given alone (table 3). This indicated that nicotinic acid partly inhibited the calorigenic action of noradrenaline. The inhibition was probable dependent on the blockade of the FFA-mobilization

Discussion

Metabolic actions of nicotinic acid. Our experiments on the rabbit showed that nicotinic acid reduced the FFA level in plasma had a slight hyperglycaemic effect and stimulated the basal oxygen consumption The hyperglycaemic effect of noradrenaline was potentiated whereas the calorigenic action was reduced As mentioned in the introduction, nicotinic acid has also other metabolic actions.

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CARLSON & ORÖ (1962) and CARLSON & NYE (1966) have suggested that the lowering effects of nicotinic acid on cholesterol triglycerides and phospholipids are a concequence of the blockade of the FFA-mobilization. This may not be the whole truth however as nicotinic acid blocks

curve was 10.3 %. From these values there was thus no indication that nicotinic acid had any blocking effect on the calorigenic action of adrenaline, most of which could be ascribed to an increased lactate metabolism.

Different results were obtained, however when the effect was calculated only for the period 0–60 min. i.e. during the infusion of adrenaline. The mean effect for the Adr curve was 11.3 % whereas the corresponding value for the Nic. ac. + Adr Nic. ac. curve was only 2.3 %. Some of this 9 % reduction may be attributed to the simultaneously observed blockade of the FFA-mobilizing effect of adrenaline, but we hesitate to ascribe the total effect to this action. The increase in the blood lactate in the Nic. ac. + Adr test could be associated with an increase of the oxygen consumption, which was 9.5 %. The difference (7.2 %) between the observed value (2.3 %) and the calculated value (9.5 %) indicated that the lactate oxidation was inhibited during the infusion of adrenaline and/or the calorigenic effect of lactate oxidation was masked by a depressant effect of adrenaline on the oxygen consumption. During the first 20 min. of infusion of adrenaline there was a significant decrease (8 %) of the oxygen consumption under the basal value in the Nic. ac. + Adr test, an effect which was not present when adrenaline was given alone. This finding indicates that the second alternative was of importence. It seems probable that the depressant effect was sustained as long as adrenaline was infused. The very marked stimulation of the oxygen consumption observed after cessation of adrenaline infusion in the Nic. ac. + Adr curve may indicate the end of this depressant effect.

The nature of this depressant effect of adrenaline on the oxygen consumption is worthy of discussion. After blockade of the metabolic action of adrenaline with adrenergic β-blockings frugs, adrenaline also induced an initial depression of the oxygen consumption (AABERG *et al* 1968). During the infusion of noradrenaline too there was a small initial depression of the oxygen consumption and a marked rise after the nor adrenaline infusion had ended (fig. 3) These findings may indicate that the initial depression and secondary rise in the oxygen consumption after adrenaline was some kind of "oxygen debt" resulting from the vasconstriction effect of adrenaline, and which was compensated for after the end of the adrenaline infusion.

The rise in the lactate content of the blood during infusion of adrenaline was increased after nicotinic acid. This indicates either that the lactate elimination was reduced or that its formation was increased by nicotinic acid. The increase in oxygen consumption during the infusion of adrenaline in the test with Nic. a. + Adr was less than that calculated for lactate oxidation, which points to the first alternative. The possibility that lactate oxidation and gluconeogenesis from lactate was

the FFA metabolism may be considered as lactate metabolism seemed to be reduced as long as the FFA plasma level was depressed by nicotinic acid (fig. 2) WEBER *et al* (1966) have suggested the FFA-level has a regulating action on the gluconeogenesis.

Summarizing we suggest that nicotinic acid initially reduced the calorigenic action of adrenaline by inhibiting the FFA mobilization and decreasing the oxidation of lactate When the stimulating effect of adrenaline on the oxygen consumption was reduced a decreasing action was unmasked which was probably related to the vasoconstriction action of adrenaline (LUNDHOLM 1949) After the end of the adrenaline infusion an oxygen debt was paid off The increased oxygen consumption partly depended on the raised lactate level of the blood partly on a postponed oxygen demand following the vasoconstriction action of adrenaline.

The FFA mobilizing action of noradrenaline was totally blocked and its calorigenic action significantly reduced by nicotinic acid This indicates that an increase in the mobilization and metabolism of FFA was of importance for part of the calorigenic action of noradrenaline. In man HAVEL *et al* (1964) calculated this part to be about one half of the total effect In the rabbit our results indicated that the FFA mobilization and metabolism were of about the same relative importance

Summary

The influence of nicotinic acid in doses of 5 15 and 100 mg/kg infused intravenously on some metabolic reactions was studied in fasted, unanaesthetized rabbits All doses of nicotinic acid reduced the plasma FFA concentration The duration of the effect increased with larger doses. The FFA-reducing effect was not influenced by blockade of adrenergic β-receptors. In a dose of 15 mg/kg nicotinic acid had a weak but significant hyperglycaemic effect which was blocked by an adrenergic β-blocking agent. The basal metabolism was also stimulated significantly (7–9 %) by all doses of nicotinic acid The FFA-mobilizing effect of adrenaline was inhibited by nicotinic acid and its calorigenic effect was partly depressed initially The effect of adrenaline on the lactate content of the blood was potentiated by nicotinic acid The FFA mobilizing effect of noradrenaline was inhibited by nicotinic acid and its calorigenic effect was reduced. The hyperglycaemic effect of noradrenaline was potentiated by nicotinic acid. It is probable that some of the metabolic effects of nicotinic acid itself and its effect on the corresponding actions of the catecholamines, may be ascribed to a combined inhibition and stimulation of the adenyl cyclase cyclic 3–5 AMP system via direct and indirect mechanisms.

Acknowledgements

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The Distribution and Elimination of Decamethonium after Single and Repeated Intravenous Injections into the Intact Rabbit

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(Received May 1 1968)

Following the intravenous injection of ^{14}C-decamethonium into bi lateral nephrectomized rabbits we observed a rapid fall of radioactivity in the blood within the first 20 minutes followed by a slower fall which was linear in a semi-logarithmic plot (BROEN CHRISTENSEN & SCHOU 1963) The initial steep fall in the decamethonium concentration in the blood probably results from the distribution of the compound in the extra cellular space and a specific uptake in the striated muscles as demonstrated by WASER & LÜTHI (1957) in the mouse diaphragm. The continued slow disappearance of decamethonium from the blood in nephrectomized rabbits is probably caused by a continued decamethonium uptake in the muscles and in the liver In rabbits the muscular uptake quantitatively plays the greatest rôle (BROEN CHRISTENSEN 1965)

The object of the present study was to examine the distribution of decamethonium after intravenous injection into intact rabbits with par ticular regard to the time course of the uptake in various muscles and its elimination from the muscles. In addition a study was carried out of the distribution following repeated intravenous injections of decamethonium In order to obtain a parameter for the biological effect of decamethonium we have measured the expiratory volume during the first 5 minutes after decamethonium administration.

Methods

Twenty one albino rabbits of both sexes with an erage weight of 2174 g (range 1930– 4 4 g) were used. A cannula was introduced into the trachea od polythene catheter into the carotid artery of the animals under light N_2O–O_2-halothane anaesthesia.

A single dose of 90 µg/kg of ^{14}C-decamethoni m bromide was i jected int the marginal vein of the ear in 17 e periments. In 4 experiments the dose wa repeated 30 mi utes after the initial injection.

The ^{14}C-decamethonium w pplied by The Radiochemical Centre, Amersham, England and had high specific activity (139 µc/mg). The radiochemical p rity of the compound w epeatedly controlled by paper chromatogr phy throughout the experimental period which lasted for 3 mo ths (the method is described by BROEN CHRISTE SEN 1967).

The expir tory vol me was mea ured by me ns of Flei h tube connected to a differential transducer and mpl fier connected to a pparatus for elect onic integration of the signals (the ppar tus and method h been described in detail by JENSE HOLM 1965). The expiratory ol me i pressed in the following a a percentage f the control alue which was determined every experiment for a period of 1 minute before the dministration of decamethonium.

Arterial blood samples wer dr via the catheter in the ca otid artery At the end of the e periment the animal was killed by i ject ng air into the ear ein. The muscle specimens were excised from the di phragm, the sternomastoid and femoral extensor quadriceps muscles. The diaphragm and sternomastoid muscles were prepared using full length fibres.

The me surement of the ^{14}C concentration in the plasma nd muscles was carried out s previously described, (BROEN CHRISTENSEN 1965) us g the liquid scintillation technique with Pack rd Tri-Carb Scintillation Spectrometer model 3003 All the results are based on the me n of duplicate determinations. The counting rate for samples with the lowest content of decamethonium wa t least 10 times as great the background counting rate. At least 5000 counts per sample were recorded

Results

The distribution and elimination following a single injection of decamethonium

In the first few minutes following the intravenous injection of 90 µg/kg decamethonium it was possible to register a moderate reduction in the expiratory volume which served as an expression the partial neuromuscular block in the respiratory muscles In the first 5 minutes after the injection the expiratory volume on an average was 88 per cent of the control value The greatest reduction in the expiratory volume occurred within the first minute A value of 83 ± 6 per cent of the control value (mean ± S E.M) was found in 9 animals.

In fig. 1 a semilogarithmic plot is shown of the course of the decamethonium concentration in the plasma following intravenous injection of 90 µg/kg. The concentration fell steeply within the first 20 minutes, then somewhat more slowly and from 60 minutes to 240 minutes after the injection, the course was practically linear The half life of decamethonium in the plasma was 48 minutes for this period

The results of the determination of decamethonium in the striated muscles measured at varying times after the decamethonium injection

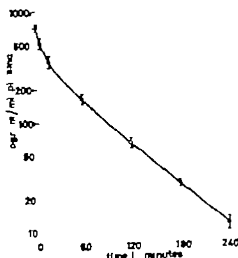


Fig. 1 Concentration of decamethonium in plasma after intravenous injection of 90 μg/kg. The decamethonium concentration is plotted on a logarithmic scale against time on an arithmetic scale. Each point is the mean of values from 4 experiments. The vertical bars indicate S.E.M.

are shown in table 1. The highest concentrations were measured 5 minutes after the injection, but there were no large changes within the first hour. The decamethonium concentration in the diaphragm and in the sterno-mastoid muscle were almost similar, i.e. approximately twice as high as the concentration in the quadriceps muscle. In this muscle there was an even fall in the values from the maximum value 5 minutes after the in-

Table 1

Concentration of decamethonium in striated muscles of the rabbit 5, 10, 20, 60 and 240 minutes after i.v. injection of 90 μg per kg.

Time in min	No. of exp.	Concentration (nanogram per g wet weight) fem. ext. quadriceps	sterno-mastoid	dia-phragm
5	2	55 (44–66)	106 (98 114)	104 (104–104)
10	3	51 (44–57)	84 (78–89)	87 (72–97)
20	3	48 (41 55)	96 (89–109)	101 (89–111)
60	5	44 ± 4	88 ± 9	99 ± 13
240	4	25 ± 1	42 ± 4	55 ± 4

Results are given as mean values ± S.E.M. Figures in brackets indicate range.

jection, whereas in the diaphragm and the sternomastoid muscle there was a slight increase in the decamethonium concentration from 10 to 20 minutes after the injection From 60 to 240 minutes after injection the content of decamethonium in all 3 muscles was reduced by approximately 50 per cent.

The distribution after repeated injection of decamethonium

In 4 experiments 90 μg/kg of decamthonium was given twice with a 30 minute interval. Two of the animals showed a reduction in the expiratory volume of approximately 50 per cent of the control value within the first minute of the second injection and were thereafter artificially ventilated, while the two other were relatively unaffected.

In a semilogarithmic plot (fig. 2) the course of the decamethonium concentration in the plasma following the second injection is parallel with the

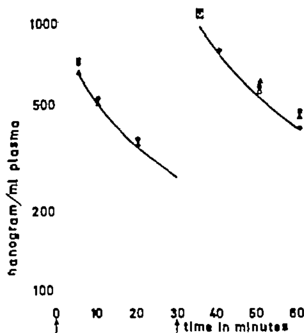


Fig. 2. Concentration of decamethonium i plasma after repeated intra enous injection of 90 μg/kg

At arrows I. Injection of 90 μg/kg decamethonium. The decamethonium concentration is plotted on a logarithmic scale against time on arithmetic scale. The curves are drawn through the mean of values from 4 experiments. The experimental esults are indicated with a special symbol for each animal (○ △ □ ●). In two experiments indicated by ○ & □ the rabbits were given artificial respiration fter the second dose.

Table 2

Concentration of decameth nium in striated muscles of the rabbit after i. injectio of two doses of 90 µg per kg with an interval of 30 minutes. The decamethonium concentration was measured 30 minutes after the second dose.

No. of exp.	Concentration (nanogram per g wet weight)		
	fem. ext. quadriceps	sterno-mastoid	dia-phragm
4	79 ± 3	14. ± 11	160 ± 13

Results are given as mean values ± S.E.M.

course after the first injection, but the curve lies at a higher level. The ratio between the mean concentration of decamethonium 5 minutes after the second injection and the mean concentration 5 minutes after the first injection ($C_{35 min.}/C_{5 min.}$) is 1 47 Similarly the ratio ($C_{40 min.}/C_{10 min.}$) = 1.54 the ratio ($C_{50 min.}/C_{20 min.}$) = 1.54 and the ratio ($C_{60 min.}/C_{30 min.}$) = 1.51

The mean concentration of decamethonium in the plasma 30 minutes after the second injection was 403 nanogram/ml. This value is almost equal to the value obtained by adding the mean concentration of decamethonium 30 minutes after the first injection (267 nanogram/ml) to the mean concentration of decamethonium 60 minutes after a single dose (162 nanogram/ml)

Table 2 shows the concentration of decamethonium in the muscles 30 minutes after the second injection. The values in all 3 muscles are approximately 50 per cent higher than the decamethonium concentration 20 and 60 minutes after a single injection (compare with table 1 from which it can be seen that these values are practically the same).

Discussion

The concentration of decamethonium in the plasma falls rapidly in the first 20 minutes after the injection, but not appreciably more rapidly than in nephrectomized animals (Brøen Christensen 1965) which must mean that the renal elimination in this period plays only a minor role in relation to the uptake in other organs particularly the muscles. The distribution phase is practically finished 1 hour after the injection and the concentra

tion of decamethonium in the plasma thereafter falls linearly in a semi logarithmic plot, as an expression of an elimination which is dominated by the renal excretion of the compound.

The decamethonium content of the muscles varies only slightly in the period from 5 to 60 minutes after the injection and there are too few observations for a statistical evaluation of the variation However the concentration of decamethonium shows a very similar course in the diaphragm and sternomastoid muscle both of which include muscle fibres along their whole length and thus definitely contain the motor end plate zones. The concentration of decamethonium falls here from 5 to 10 minutes and then increases from 10 to 20 minutes after the injection, after which it falls slowly The observations are comparable with the view that there are two accumulation maxima, an initial one which is as an expression of the binding of decamethonium to the receptors in the motor end plate and a secondary one which might be due to the penetration of decamethonium into the muscle fibres Waser's autoradiographic studies of the mouse diaphragm showed accumulation of radioactivity in the motor end plate zone 1–2 minutes after the intravenous injection of ^{14}C-decamethonium The width of the radioactive zone diminished from 2 to 10 minutes after the injection, after which it increased again (WASER 1963)

We have found the same relation between the decamethonium concentration in the diaphragm and in the muscles of the extremities as that found by LÜTHI & WASER (1965) in studies on cats given 200 µg/kg of decamethonium intravenously

MANERY & HASTINGS (1939) on the basis of chloride analyses have estimated that extracellular volumes in the diaphragm and muscles of the extremities of rabbits are respectively 25 and 13 per cent of the wet muscle weight Assuming that a diffusion equilibrium is present between decamethonium in the plasma and in the extracellular phase of the muscle 60 minutes after injection, we have calculated the amount of free diffusible decamethonium in the extracellular space to be approximately half of the total decamethonium content of the muscles at this time.

In the period 60 to 24 minutes after the injection, the total decamethonium content in the muscles is reduced by approximately 50 per cent (table 1) whilst the decamethonium concentration in the plasma falls from 162 to 12 nanogram/ml. Hence the amount of decamethonium which remains in the muscles after 4 hours is mainly bound to the muscle cells. The amount of bound decamethonium is thus practically unchanged from 1 to 4 hours after injection, whereas the major portion of the decamethonium in the extracellular phase is eliminated

The accumulation of decamthonium in the muscles has given rise to the

question whether it can penetrate into the muscle cells. The autoradiographic studies of CREESE & MACLAGAN (1967) support the assumption that decamethonium can enter the fibres of the rat diaphragm. The studies also showed that muscles, which had taken up decamethonium retained this for a very long time The same conclusion was arrived at by TAYLOR, DIXON CREESE & CASE (1967) who also studied the uptake of decamethonium in the rat diaphragm, but by another technique. In these experiments it was possible to demonstrate a considerable retention of decamethonium 10 days after its administration.

The parallel course of the curves for the concentration of decamethonium in the plasma after the first and the second injection of decamethonium (fig. 2) might be taken as evidence that the distribution and elimination pattern is the same after the second as after the first injection. The results of the decamethonium estimation in the muscles 30 minutes after the second dose (table 2) are consistent with this view WASER (1962) has demonstrated that the decamethonium uptake in the mouse diaphragm after a single administration is directly proportional to the dose in a range from 0.2 to 2.2 µg/g, which suggests that the uptake process is controlled by simple physico-chemical laws.

In 2 of 4 experiments the effect of the second dose was greater than the effect of the first dose. ALDERSON & MACLAGAN (1964) in cat experiments found that the sensitivity of the diaphragm to decamethonium was reduced when the dose was repeated after an interval of 1 hour The intervals between the decamethonium injections must, however have considerable influence on the effect, partly because of differences in the plasma level of decamethonium and partly because of variation in the sensitivity of the muscles to decamethonium Decamethonium blocks the neuromuscular transmission by a dual mode of action which includes an initial depolarization block followed by a competitive block (JEWEL & ZAIMIS 1954). These two types of block counteract each other and the sensitivity of the muscles to repeated doses of decamethonium will therefore depend on the type of block phase in which the muscles are at the time of renewed administration.

Summary

1 An account is given of the time course of decamethonium concentration in the plasma and in striated muscles following intravenous injection of 90 µg/kg of decamethonium into rabbits.

2. The elimination of decamethonium from the plasma is initially very rapid owing to its distribution in the extracellular phase and the accumulation in striated muscles. From 1 hour after the injection the con

centration of decamethonium in the plasma falls exponentially which is taken as an expression of the renal excretion of the compound. The half life of decamethonium in the plasma was estimated to be 48 minutes.

3 The decamethonium uptake in the muscles is a rapid process that reaches its maximum values within 5 minutes after injection. The elimination of decamethonium from the muscles is a slow process which does not run parallel with the elimination of decamethonium from the plasma. The total content of decamethonium in the muscles is reduced by 50 per cent from 1 to 4 hours after injection but the decamethonium bound to the muscle cells is not appreciably reduced during the same period.

4 In 4 experiments in which 90 µg/kg of decamethonium was given twice with a 30 minute interval, the time course of concentration of decamethonium in the plasma was determined. In a semilogarithmic plot the course of decamethonium concentration in the plasma following the second injection was found to be parallel with the course after the first injection. These data might be taken as evidence that the distribution and elimination pattern is the same after the second as after the first dose.

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Influence of Antirheumatic Agents on the Release of Histamine from Sensitized Rat Peritoneal Mast Cells
II Antibody Production

By

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(Received July 1 1968)

When peritoneal mast cells from sensitized rats are incubated *in vitro* with a specific antigen histamine is released (UVNÄS & THON 1959 MOTA & ISHII 1960 PERERA & MONGAR 1963 NORN 1965) If towards the termination of the sensitization period the rats are treated with hydrocortisone phenylbutazone, or sodium aurothiosulphate, the release of histamine is inhibited (NORN 1965).

The purpose of the present study was to investigate whether the ability of these agents to inhibit histamine release is due to an inhibition of production of the antibodies which cause the release of histamine from the mast cells. The antibody produced is present partly bound in tissues and partly circulating in the blood The concentration of circulating antibody can be measured indirectly by the release of histamine elicited in rats passively sensitized with serum from the rats pretreated with antirheumatic agents Reduced release of histamine from the mast cells of the passively sensitized rats, when these cells are exposed to antigen *in vitro* would thus indicate whether the antirheumatic agents had inhibited the production of the antibody

Materials and Methods

Non-sensitized rat

Female albino rats (strain from Leo Pharmaceutical Products, Copenhagen) weighing 150–200 g were used.

Sensitized rats

The above-mentioned rats were sensitized to horse serum as described by NORN (1965).

Treatment of sensitized rats with antirheumatic agent

During the last 3 days of the 3 week sensitization period the rats were given daily subcutaneous injections of either 100 mg/kg hydrocortisone, 200 mg/kg phenylbutazone, or 25 mg/kg sodium aurothiosulphate (test rats). The dose was administered in volume of 10 ml/kg body weight, the last-mentioned substance being administered in modified Tyrode solution and the other substances i suspensions. The suspensio medium consisted of 1 part Acacia Mucilage (U.S.P 47) and 6 parts modified Tyrode solution. The control rats were treated in the same way with modified Tyrode solution or the named suspension med um. Each test and control group comprised 8 rats. Two hours after the last injection, the rats were killed by exsanguination from the carotid arteries. The blood was left to stand for few hours at 4 The clot was then detached and the serum withdrawn. The serum from each rat was dialyzed to remove ny content f antirheumatic substance as follows 2 3 ml serum was put into a dialysis tube (Visking seamless cellulose tubing, average pore radius f 24 Å) and placed in a cylinder with 33 ml modified Tyrode solution. The cylinder was gently rocked, and the outer fluid changed at intervals of 1 2, 11 nd 2 hours respectively Two ml f the dialysed serum was mixed with 6.0 ml modified Tyrode solution.

Passive sensitization by serum from antirheumatic-treated donors

Seven ml f each of the above-mentioned serum dil tions were injected intraperitoneally into non-sensitized rats, each rat receiving first 4 ml and then, 2.5 hours later the remaini g 3 ml. Fortyeight hours later a suspension of peritoneal cells was emoved from each of the passively sensitized rats as described by NORN (1967b). From each of these suspensions 3.50 ml samples were incubated with 250 µl horse serum (unactivated by heating to 56 for 30 min.) at 37 for 30 min. The amount f released histamine was then determined as the per cent of the total histamine content of the specimen (NORN 1967) no correction being made for the small amount of histamine released by the mechanical manipulation (table 1).

Passive sensitization of rats pre-treated with hydrocortisone

Serum was collected from sensitized rats and pooled The pooled serum was then mixed with a 3 times larger volume of modified Tyrode solution, and 7.0 ml of this mixture injected intraperitoneally into each of the non-sensitized rats as mentioned bove These rats received a daily subcutaneous injection of either hydrocortisone, suspended th named medium (test group) or of the medium alone (control group) The treatment was continued either for 6 days, starting 3 days before the passive sensitization, or for 3 days, starting at the same time as the passive sensitization. The dosage is listed i table 2. Each test and control group comprised 8 rats. Fortyeight hours after the passive sensitization, e 3 hours after the last injection of hydrocortisone, suspension of peritoneal cells was removed from all the rats and incubated with horse serum as stated above. T ble 2 gives the amount of released histamine in per cent of th total histamine content of the specimen.

Results

Passive sensitization with serum from antirheumatic treated donors

From the table 1 it can be seen that the release of histamine in peritoneal cell suspensions from rats passively sensitized with serum from sensitized rats did not change when the donors were pre treated with hydrocortis

Tabel 1

Influence of treatment with antirheumatic agents of actively sensitized donors on the histamine release from peritoneal mast cells from passively sensitized recipients. Each group comprised eight animals and the figures represent the mean values and the standard error of the mean

Pre-treatment of donors for 3 days. Daily doses	Histamine release in per cent by recipients	
	Control	Test
Hydrocortisone 100 mg/kg	[a]73 ± 6	74 ± 7
	57 ± 8	[a]64 ± 9
Phenylbutazone 200 mg/kg	34 ± 7	32 ± 8
Sodium aurothiosulphate 25 mg/kg	[a]49 ± 7	39 ± 6

[a] P > 0.1 by t-test.

phenylbutazone, or sodium aurothiosulphate respectively. During treatment with phenylbutazone 2 out of 10 rats died and a few animals had diarrhoea. The other two substances did not seem to affect the rats.

Passive sensitization of rats pre-treated with hydrocortisone

Pre-treatment of passively sensitized rats with hydrocortisone significantly inhibited the release of histamine in their peritoneal cell suspensions (table 2). This inhibition ranged from 60–80 % ($P < 0.001$) when the treatment was started 3 days before the passive sensitization and was around 30 % ($P < 0.01$) when it was started at the same time as the passive sensitization.

Discussion

Peritoneal mast cells from actively sensitized rats release histamine when incubated *in vitro* with specific antigen. This release is significantly reduced if the rats are treated, during the last 3 days of the sensitization period, with daily subcutaneous injections of either 100 mg/kg hydrocortisone, 200 mg/kg phenylbutazone, or 25 mg/kg sodium aurothiosulphate (NORN 1965). These findings were made in experiments on male rats, but subsequent experiments on female rats gave the same results (NORN, unpublished).

In the present study it was investigated whether the reduced release of histamine is due to inhibition of antibody production. Since the con-

Table 2

Influence of hydrocortisone treatment of passively sensitized recipients on th histamine release from their peritoneal mast cells. Each group comprised eight nimals and the figures represent the mean values and the standard error f the mean.

Hydrocortisone treatment of recipients	Histamine release in per cent		% Inhibition
	Control	Test	
6 days 50 mg/kg daily	51 $\pm$ 7	21 $\pm$ 6	59
	[b]43 $\pm$ 6	[b]7 $\pm$ 2	84
3 days; 100 mg/kg daily	[c]73 $\pm$ 3	[c]53 $\pm$ 5	27

a–c $P < 0.01$ by t-test.

centration of the antibody in the serum cannot be determined directly by precipitation (Mota 1963 & 1964 Binaghi *et al.* 1964), it was assessed indirectly by observing the ability of the serum to bring about passive sensitization in non-sensitized rats. The degree of passive sensitization was measured as the release of histamine which occurred when peritoneal cells from the passively sensitized rats were incubated *in vitro* with specific antigen.

The experiment disclosed that the histamine release was the same whether the recipients had received serum from antirheumatic-treated donors or from untreated donors (table 1). Thus, the demonstrated reduction in histamine release from the mast cells of an actively sensitized rat treated with either hydrocortisone, phenylbutazone, or sodium aurothiosulphate (Norn 1965) cannot be explained by an inhibition of the production of those antibodies which bring about histamine release The reduction must, therefore, be due to an inhibition either of the antigen-antibody reaction or of the enzymatic processes initiated in the mast cell during the antigen-antibody reaction (Mota & Ishii 1960 Uvnäs 1962) Both possibilities were confirmed, as far as hydrocortisone was concerned, by the finding that treatment of the recipients with this substance inhibited the passive sensitization (table 2). The greatest inhibition (60–80 %) was obtained when the administration of hydrocortisone was started a few days before the passive sensitization.

Summary

Serum from actively sensitized rats elicits passive sensitization in non-sensitized rats which can be measured by the extent of the histamine release which takes place when the peritoneal cells of the recipient are

incubated *in vitro* with specific antigen. This passive sensitization is not reduced when the donors have been treated towards the end of the sensitization period with hydrocortisone, phenylbutazone or sodium aurothiosulphate. Hence, the treatment of the actively sensitized rats cannot have inhibited the antibody production by these animals. In other words, the reduction in histamine release from the peritoneal mast cells of actively sensitized rats which has been demonstrated previously must therefore be due to an inhibition either of the antigen antibody reaction or of the enzymatic processes.

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Effect of Thyroxine on Thioacetamide Hepatotoxicity

By

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Thyroxine treatment of rats has been shown to augment carbon tetra chloride hepatotoxicity (CALVERT & BRODY 1961) This was attributed to the increased oxygen need of the liver tissue of such animals as well as to an excessive liberation of catecholamines from their stores.

The liver damage induced by thioacetamide resembles, at least in some respects, that of carbon tetrachloride, e.g. protection by antihistaminic drugs (GALLAGHER *et al* 1956) It was, therefore, decided to study the effect of thyroxine on acute thioacetamide poisoning.

Materials and Methods

Stock albino rats, weighing 150–200 g were used. Thyroxine sodium, 0.2 mg, was given orally for five successive days. The animals received thioacetamide or carbon tetrachloride on the 6th day Carbon tetrachloride, 1.25 ml/kg body wt. was given by stomach tube, mixed with an equal amount of liquid paraffin. Thioacetamide was administered intra peritoneally as an aqueous solution in the dose of 200 mg/kg. The body temperature of the animals was recorded with rectal thermometer

Hepatic function was studied 4 hours after the administration f thioacetamide or carbon tetrachloride. Sleeping time was noted after intraperitoneal injection of 80 mg/kg of benzobarbitone sodium. The time interval between the loss and regain of righting reflex was taken as the sleeping time. Bromsulphthalein was injected intraperitoneally as an aqueous solution in the dose of 0.05 mg/g body wt. (CASALS & OLITEKY 1946). Blood was collected thirty minutes later from the neck vessels under light ether anaesthesia and the concentration of bromsulphthalein in the serum was estimated colorimetrically after alkalinisation (WOOTTON 1964).

The liver was removed, blotted and one gram of liver tissue weighed on torsion balance and homogenised immediately in ice-cold sucrose solution (0.25 M). Estimation of succinic dehydrogenase activity was made colorimetrically by the method of KUN & ABOOD (1949) using triphenyltetrazolium chloride (TTC). The activity was expressed as µg of TTC reduced/mg of liver tissue.

A piece of liver tissue was removed, fixed in 10 % formalin in saline and blocked in paraffin. Liver histology was studied after staining with haematoxylin and eosin.

Statistical comparisons were made using Student' "t test for camparing means.

Table 1

Body Temperature of the animals.

	No of animals	Body Temperature (°C) ± S.D At 0 hr	At 24 hr	P
1 Blank control	9	36.95 ± 0.10	37.00 0.12	
				1 Vs 2
2. Thyroxine treatment only	9	38.20 ± 0.11	38.22 ± 0.11	P < 0.01
3 Carbon tetrachloride	9	36.81 ± 0.09[1]	36.91 ± 0.19[1]	
4 Thyroxine + carbontetrachloride	9	38.21 ± 0.12[2]	38.29 ± 0.1[illegible][2]	3 V 4 P < 0.01
5 Thioacetamide	9	36.88 ± 0.11[1]	36.87 ± 0.10[1]	
				5 Vs 6
6. Thyroxine + thioacetamide	9	38.19 ± 0.11[2]	38.[illegible]1 ± 0.12[2]	P < 0.01

[1] P > 0.1
[2] P > 0.1

Results

The body temperature of rats treated with carbon tetrachloride or thioacetamide showed no significant change at the end of 24 hours. Pretreatment with thyroxine raised the body temperature by over 1° and treatment of these animals with carbon tetrachloride or thioacetamide did not further raise their temperature (table 1).

Table 2

Hexobarbitone sleeping time.

	No of animals	Sleeping time in min. ± S.D 15 days before treatment	24 hr after treatment	P
1 Blank control	9	19.2 6.27	15.8 ± 5.78	
				1 Vs 2
2. Thyroxine treatment only	9	[illegible]0.4 3.72	19.5 ± 4.32	P > 0.1
3 Carbon tetrachloride	10	18.4 ± 7.81	80.3 ± [illegible]3.04	
4 Thyroxine + carbon tetrachloride	8	16.6 ± 4.32	128.7 ± 31.05	3 Vs 4 P < 0.01
5 Thioacetamid	9	22.7 ± 5.34	71.5 ± 15.27	
				5 Vs 6
6. Thyroxine + thioacetamide	9	19.9 ± 3.23	114.8 ± [illegible]6.7[illegible]	P < 0.001

Table 3

Bromsulphthalein excretion.

	No of animals	Serum level of bromsulphthalein (mg %) ± S.D	P
1 Blank control	9	0.42 ± 0.02	
			1 V 2
2. Thyroxine treatment only	9	0.51 ± 0.04	P > 0.1
3. Carbon tetrachloride	9	2.57 ± 0.42	
			3 Vs 4
4 Thyroxine + carbon tetrachloride	8	3.14 ± 0.37	P < 0.01
5 Thioacetamide	9	2.40 ± 0.39	
			5 V 6
6. Thyroxine + thioacetamide	9	3.28 ± 0.50	P < 0.001

Thyroxine treatment alone did not significantly alter the hexobarbitone sleeping time, bromsulphthalein excretion or hepatic succinic dehydrogenase activity The livers of such animals also showed no histological changes.

Rats pretreated with thyroxine showed a more marked prolongation of hexobarbitone sleeping time after treatment with carbon tetrachloride or thioacetamide than those treated with the toxin alone (table 2)

Table 4

Succinic Dehydrogenase Activity of the liver

	No. of animals	Succinic dehydrogenase activity (μg f TTC reduced/mg of liver tissue) ± S.D	P
1 Blank control	6	1.92 ± 0.14	
			1 V 2
2. Thyroxine treatment only	9	1.89 ± 0.12	P > 0.1
3. Carbon tetrachloride	9	1.02 ± 0.06	
			3 V 4
4 Thyroxine + carbon tetrachloride.	8	0.87 ± 0.04	P < 0.001
5 Thioacetamide.	8	1.18 ± 0.08	
			5 V 6
6. Thyroxine + thioacetamide	8	0.83 ± 0.05	P < 0.001



Fig 1 Rat Liver 4 hours after the administratio of carbon tetrachloride. Haemato ylin and eosin.

Significantly higher levels of bromsulphthalein were found in the serum 30 minutes after injection of the dye in the thyroxine pretreated animals than in those receiving carbon tetrachloride or thioacetamide alone (table 3)

Impairment of hepatic succinic dehydrogenase activity was also more marked after thyroxine pretreatment (table 4).

Histologically the livers of rats treated with carbon tetrachloride showed centrolobular necrosis, surrounded in turn by an area of cells showing fatty change and hydropic degeneration in the midzone and a zone of intact cells around the portal tracts (fig. 1)

Thioacetamide produced essentially similar changes, i.e marked centrolobular necrosis with sparing of the periportal cells, but the region of cells showing fatty change and hydropic degeneration in the midzone was indistinct or absent (fig. 3)

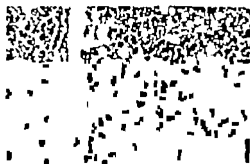


Fig. 2. Rat Pretreated with thyroxine Liver 24 hours fter administration of carbon tetrachloride. Haematoxylin and eosin.



Fig. 3 Rat Liver 24 hours after the administration of thioacetamide. Haematoxylin and eosin.

Pretreatment with thyroxine produced qualitatively the same changes after administration of carbon tetrachloride or thioacetamide but the extent of centrolobular necrosis with both the poisons was more marked (fig. 2 and 4).

Discussion

Many similarities have been observed between carbon tetrachloride and thioacetamide with regard to their hepatotoxicity The early loss of cytoplasmic basophilia, the development of oedema channels and centrolobular necrosis are features common to both (GUPTA 1956). Both substances are protected by antihistaminic drugs and adrenalectomy (REES *et al* 1961) and are associated with early dislocation of ribosome from the membranes of the endoplasmic reticulum and altered aminoacid incorporating capacity in vitro (BARKER *et al* 1963). The present finding



Fig. 4. Rat pretreated with thyroxine. Liver: 24 hours after administration of thioacetamide. Haematoxylin and eosin.

that thyroxine pretreatment increases the susceptibility of rats to the hepatotoxic actions of thioacetamide as well as carbon tetrachloride points to another similarity between these two poisons.

However differences have also been observed between these two poisons. REES *et al* (1961) found that thioacetamide causes hepatic necrosis without fatty change, though lipid accumulation could be demonstrated by GUPTA (1956). Our findings are in agreement with REES *et al* (1961) Mitochondrial damage has been found to occur earlier in thioacetamide poisoning and is "possibly greater than in carbon tetrachloride poisoning" (REES & SINHA 1960). Thioacetamide has also a stimulating action on hepatic malic dehydrogenase (REES & SINHA 1960).

The augmentation of carbon tetrachloride toxicity by thyroxine was explained by CALVERT & BRODY (1961) on the basis of their catecholamine hypothesis Thyroxine pretreatment increased the release of catecholamines from the adrenals, reduced the monoamine oxidase activity of the liver and increased the oxygen requirement of the liver tissue. These factors together result in greater susceptibility of the liver cells to the hypoxia caused by sympathetic overactivity Whether such an explanation can be applied in the case of thioacetamide is not yet known. The catecholamine hypothesis of carbon tetrachloride hepatotoxicity cannot explain all the aspects of the action of this toxic agent Recently attention has been directed to alteration of protein synthesis and lipoperoxidation mechanisms as the cause of hepatic damage by carbon tetrachloride (RECKNAGEL 1967) Hence an alternative explanation accounting for the effects of both carbon tetrachloride and thioacetamide should be sought.

Hypothermia has been shown to protect against carbon tetrachloride toxicity (LARSON & PLAA 1965) In the present study thyroxine pretreatment raised the body temperature by 1 which may be the reason for the increased toxicity of carbon tetrachloride. Though no reports have appeared in the literature about the effect of body temperature on thioacetamide toxicity it would appear that the latter follows a similar pattern to that of carbon tetrachloride. Thyroxine may be increasing the energy requirements of the liver cell an effect which may make the cell more vulnerable to toxic attack irrespective of the mechanism of the latter Studies on the effect of thyroxine on several other hepatotoxins may help to explain this point.

Summary

Pretreating rats with thyroxine for five days before challenging with carbon tetrachloride or thioacetamide resulted in an increase in the liver damage. This was assessed by hexobarbitone sleeping time, bromsulph-

thalein excretion and liver succinic dehydrogenase activity The histolog ical findings were consistent with the results obtained

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Effects of Formaldehyde and Formic Acid on Histamine Metabolism in Sheep

By

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When a mixture of histamine and formaldehyde is heated in acid solution a bicyclic compound is formed (FRÄNKEL & ZEIMER 1920). Further addition of formaldehyde to histamine solutions greatly reduces the bio-activity of the latter substance (DALE & DUDLEY 1921 KENDALL 1927 BEST & MCHENRY 1930).

NEUMARK, BONDI & VOLCANI (1964) found that histamine and formaldehyde given simultaneously by mouth to small ruminants resulted in toxic manifestations, whereas no effect was observed when the substances were given separately They stated that the toxicity was due to increased absorption of formaldehyde caused by histamine. This explanation, however seemed somewhat doubtful since formaldehyde injected intravenously in large amounts has been found to be non-toxic in rabbits (BARRES 1960a & b) Small amounts of histamine, on the other hand, are toxic when given intravenously to sheep (DOUGHERTY 1942 DUNCAN 1954) It therefore seemed possible that the effect observed by NEUMARK *et al* (1964) was mainly due to potentiation of the histamine effects by formaldehyde Since such an assumption was apparently contradictory to the above mentioned observation that the histamine-formaldehyde complex possesses little bio-activity it was considered desirable to examine the effects of formaldehyde on the metabolism of histamine in sheep

The fact that HCHO is metabolized to CO via HCOOH as well as the observations of increased toxicity of histamine when given by mouth together with HCOOH (NEUMARK *et al* 1964) made it desirable also to examine whether HCOOH affects histamine metabolism

Materials and Methods

Substances

Histamine dihydrochloride and Histamine diphosphate were obtained from Nutritional Biochemicals Corp. Cleveland, Ohio, U.S.A.

Pipsyl chloride (p-iodobenzenesulfonyl chloride) was purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

^{14}C-histamine dihydrochloride labelled i th ring- -positio was obtained from The Radiochemical Centre, Amersham, England. The spec. act. was 30.5 mc/mmole.

Antihistamine, Allergin ® (Diphenhydramine hydrochloride) was bought from Nyegaard & Co. A/S, Oslo Norway

Formaldehyde solution 35 per cent (w/w) p.a. and *Formic acid solution* 90 per cent (w/w) p.a. were obtained from Merck A G Darmstadt, Germany

Amberlit Resin CG50 chromatographic grade 100–200 mesh f m the British Drug Houses LTD England, was used.

Three male sheep of the Dala breed were used for the experiments. They were fed diet of hay and concentrates twice daily Urin was collected qua titatively in plastic bottles containing 250 ml of N-HCl providing pH lower tha 2 as described lsewhere (SIAASTAD to be published) The urine was f ozen and stored at −20 until extraction f histamine.

Loading was done by administering 1 litre f water containing histamine or formaldehyde or both by stomach t be. When both substances were given simultaneously they were mixed before administration.

Preparation of the urine for biological assa of free and conjugated histamine

Free histamine was extracted from urine by means f weak acidic catio exchange resin, Amberlite CG50, according to O TEL, MARSH & SJOERDSMA (1962). Some modifica tions were introduced and the procedure is briefly as follows

Samples of 4-hour urines were adjusted to pH 7.5 with 10 N N OH Sixty ml aliquots were thoroughly mixed with 30 ml 0 15 M sodium phosphate buffer (pH 7 5). Forty ml of the buffer-urine mixture were passed through the column (60 × 12 mm, pproximately 0.3 ml per min.), and then 50 ml of 0.5 M sodium acetate buffer pH 6.5 was percolated through the column. Histamine was eluted by passing N-HCl through the column (0 2 ml per min.) until the pH of the effluent had changed towards the acid side From this poi t on elutio was continued with 10 ml f N/100 HCl and collection of the luate for histamine determination started

The eluates were stored at +4 for a period not longer than 5 days. The histamine concentrations of the neutralized eluates were then determined on the isolated guinea-pig ileum, suspended in an organ bath (6 ml) containing Tyrode solution (37°) with tropine (0 05 µg/ml) and glucose (1 g/l).

Conjugated histamine Fifty ml samples of urine were precipitated with acetone nd hydrolysed under reflux with 10 N HCl for 2 hours according to D NER & PERNOW (1956). The hydrolysates were concentrated to dryness vacuum. Th residues were dissolved i distilled water filtered, adjusted to pH 7.5 and made up to final vol me of 50 ml. These solutions were mixed with sodium-phosphate buffer (25 ml, 0.5 M pH 7.5) nd passed through the ion-exchange columns (120 × 12 mm). After washing with sodium acetate buffer (100 ml, 0.5 M pH 6.5) the procedure was as for free histamine. The values obtained represent total histamine (free + conjugated).

The mea recoveries of histamine diphosphate and N-acetyl histamine (believed to be identical to conjugated histamine, TABOR & MOSETTIG, 1949) were 84 7 ± 11 7 (S.D 15 expts.) and 76.5 ± 4.2 (8 expts.) „ respectively

The values for both free and conjugated histamine in the present paper represent the mean of duplicate extractions and are given in terms of the base.

To check that the contracting activity of the eluates was due to the presence of histamine, an antihistamine diphenhydramine hydrochloride (Allergin ®) was occasionally added to the organ bath. Further paper chromatography was used to confirm that the increased bio-activity in the urine of HCHO-treated sheep was accompanied by a corresponding increase in the amount of urinary free histamine.

Incubation of liver and kidney tissue

Homogenates of kidney and liver tissue of sheep were prepared as previously described (Sjaastad 1967a). To 4 ml of homogenate (corresponding to 200 mg of tissue) formaldehyde or formic acid (0.1 ml), carrier histamine (in 0.1 ml) and ^{14}C-histamine (in 0.05 ml) were added (final volume 4.25 ml). Erlenmeyer flasks (25 ml) containing the incubation mixtures were "gassed" with a mixture of O_2-CO_2 (95:5) and incubated at 37° under continuous shaking for 1½ hours. The incubation was terminated by heating the incubation mixture to 100° and by acidifying it with 1 ml of N HCl.

The amounts of histamine left after incubation were determined by one of two methods 1) bioassay 2) radioautography followed by counting of the radioactive area corresponding to histamine in a Beckman flow counter (Low beta II). The amount of imidazoleacetic acid (ImAA) formed during incubation was determined by counting of the radioactive area corresponding to ImAA.

Two-dimensional paper chromatography was carried out with n-butanol-acetic acid-water (4:1:1) and n-butanol saturated with 20 % NH_3. Imidazoleacetic acid and 1-methyl-4-imidazoleacetic acid are not separated by this procedure. By using other solvent systems it was found, however, that the samples which were subjected to chromatography did not contain detectable amounts of 1-methyl-4-imidazoleacetic acid.

Results

Effect of HCHO and HCOOH on the urinary excretion of histamine

HCOOH in amount of 0.2 mole or less, when given by mouth simultaneously with 0.3 g of histamine base, did not influence the urinary excretion of free histamine.

The fraction recovered as conjugated histamine seemed to be lowered by HCOOH. Toxic manifestations apart from loss of appetite for a few hours, in some instances, were not observed.

HCHO. When HCHO and histamine (0.3 g base) were given simultaneously by mouth, it was found that HCHO in amounts of 0.02 mole or more increased the urinary excretion of biologically active histamine (fig. 1a). When 0.2 mole HCHO and 0.3 g histamine were given (6 expts., 3 sheep) 3300 µg corresponding to 1.1 per cent of the administered dose was on an average recovered in the urine as biologically active histamine during the first 24 hours (range 0.4–2.6 per cent). The corresponding percentage for the same 3 sheep when loaded with histamine only was 0.03 (range 0.01–0.07). Furthermore, toxic manifestations appearing

Fig. 1a

Fig. 1b

Fig. 1 & b. Effect of formaldehyde on the urinary excretion of free (1) and conjugated histamine (1 b) when histamine (0.3 g base) and formaldehyde are given simultaneously by mouth to a sheep. The hatched columns represent the amount of free or conjugated histamine excreted 24-hours before loading.

within 1–2 minutes of administration and lasting for 2–4 days, were invariably observed in these instances. When 0.2 mole of HCHO was given alone (2 expts.), the urinary excretions of free histamine were 19 and 15 µg/24 hr., respectively In the last mentioned experiments signs of intoxication were not observed.

In experiments in which 0.2 mole of HCHO and 0.3 g of histamine were given the excretion of histamine in conjugated form in the urine increased considerably (range 5600–22000 µg/24 hr as compared with 1800–3800 µg/24 hr when histamine only was given). When 0.02 mole or less of HCHO was given the urinary excretion of histamine in conjugated form did not differ significantly from that found in experiments in which histamine alone was given (fig. 1b). In one experiment, in which 0.4 mole HCHO and 1.0 g histamine were given, biologically active substance(s) equivalent to 220 mg of histamine base were excreted in the urine during the first 96 hrs. subsequent to administration. In this experiment complete anuria *was observed during the first 30 hours after administration.* Moreover shortly after administration the animal showed hyperpnoea, increased pulse rate and anorexia. The next day the animal was unable to get up. Because of the severe toxic effect the sheep was repeatedly treated with antihistamine (Allergin ® 200 mg × 5). The treatment seemed to have a temporary benificial effect on the animal.

In the present experiments the symptoms of intoxication gradually decreased during consecutive administrations of histamine and HCHO to the same animal (0.3 g and 0.2 mole, respectively). The reduced toxicity was not associated with any decrease in the urinary excretion of biologically active histamine.

Intravenous injection of HCHO

In one sheep 105 ml of 10^{-1} M HCHO were injected into the jugular vein during a period of 135 min. Histamine (0.3 g) was given by mouth at the start of the HCHO infusion. The urinary excretion of both free and conjugated histamine (152 and 2530 µg/24 hr, respectively) were within the control range (fig. 1a & b). No signs of intoxication were observed in this experiment.

Incubation of liver and kidney tissue

HCOOH in concentrations of 5×10^{-3} M or higher inhibited the inactivation of histamine in liver and kidney tissue (fig. 2). At a concentration of 10^{-2} M histamine inactivation was completely abolished.

HCHO. An attempt was made to demonstrate a possible inhibition of HCHO by means of the isotope dilution technique since histamine mixed with HCHO could not be adequately determined by bioassay. Inhibition seemed to occur at concentrations between 10^{-4} and 10^{-3} M. However, HCHO in high concentrations disturbed the formation of crystals and at a concentration of 10^{-1} M crystals could not be obtained. It

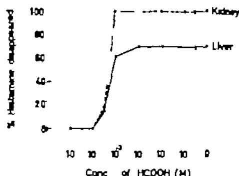


Fig. 2. Inhibition of histamine degradation by HCOOH in kidney and liver homogenates. Amount of histamine added 250 µg histamine diphosphate. Amount f tissue 200 mg. Final volume 4.5 ml. The concentration f histamine left after incubation is determined by bioassay

was demonstrated by paper chromatography that the formation of ImAA decreased when the HCHO concentration was increased from 10^{-4} to 10^{-2} M The decrease in the formation of ImAA was accompanied by a concurrent increase in the amount of unmetabolized histamine (histamine + histamine HCHO-complex) (fig. 3) An unidentified radioactive substance (R_f 0.26/0.56) of considerable quantitative importance was observed on the chromatograms.

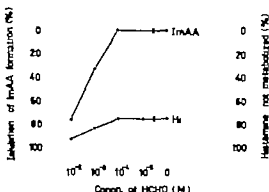


Fig. 3. The effect of formaldehyde on the histamine metabolism in kidney homogenates. Amount of histamine added 1000 µg histamine diphosphate. Amount of tissue 200 mg. Final volume 4.25 ml. The amounts of histamine and ImAA at the end of the incubation period were determined by radioautography followed by counting of the radioactive area corresponding to histamine and ImAA.

Discussion

It is known that HCHO inhibits several enzymes. The present study has revealed that HCHO in fairly low concentrations also inhibits the histamine inactivation in liver and kidney homogenates of sheep. This effect is probably due to inhibition of the oxidative deamination which is the major pathway for histamine inactivation in sheep liver and kidney tissue (SJAASTAD 1967a & b). This hypothesis is supported by the fact that the decreases in histamine inactivation in HCHO-treated tissue incubates was accompanied by a corresponding decrease in the formation of imidazole acetic acid. The present study further demonstrates that HCOOH also inhibits tissue inactivation of histamine *in vitro*. The fact that higher concentrations of HCOOH than of HCHO are needed to obtain an inhibition in the *in vitro* inactivations of histamine makes it improbable, however, that the effect of HCHO is due to transfer to HCOOH.

The toxic manifestations when histamine was given together with HCHO were in the present study associated with increased urinary excretion of histamine-like activity. But since HCHO given intravenously does not increase the toxicity of orally administered histamine, the increased toxicity when both substances were given by mouth can hardly be explained by an inhibition of the tissue inactivation of histamine.

It has been demonstrated that histamine metabolites (N-methylated histamine) possessing biological activity are normally present in the urine of humans (KAPELLER-ADLER & IGGO 1957). From paper chromatography it was evident, however, that the increased biological activity in the urine subsequent to oral administration of histamine and formaldehyde is mainly or entirely due to increased amounts of free histamine.

The large urinary excretion of free histamine after oral administration of histamine-formaldehyde and the fact that relative large doses of formaldehyde were found to be non toxic when injected intravenously strongly indicate that the toxic manifestations observed in the present study are due to histamine rather than formaldehyde.

The present study demonstrates that the fraction of orally administered histamine excreted as urinary conjugated histamine is increments by large amounts of HCHO given simultaneously. No experiments were, however, carried out in order to establish whether the increase in conjugated histamine represent N-acetylhistamine, which is believed to be identical with the conjugated histamine normally present in the urine (TABOR & MOSETTIG 1949).

There are several possible explanations for the observed toxicity as well as the increased urinary excretion of histamine when the substance is given

by mouth together with HCHO 1) It has been shown that histamine is poorly absorbed from the forestomach of sheep (SJAASTAD & KAY to be published). It has further been demonstrated that less than 10 per cent of orally administered histamine reaches the upper part of the duodenum in a biologically active state (SJAASTAD & KAY to be published) The major part of the remaining 90 per cent is probably degraded by the microflora in the forestomach. It is possible that HCHO interferes with the bacterial inactivation of histamine in the forestomach and thereby increases the amount of histamine gaining access to the intestines from where histamine has been found to be quite rapidly absorbed (SJAASTAD to be published). The validity of this hypothesis could obviously be studied by comparing the rate of disappearance of histamine from the fore-stomach when histamine is given alone, with that found when the substance is given together with formaldehyde. But since the histamine-formaldehyde complex does not possess any bioactivity and furthermore since formaldehyde interferes with the formation of histamine-pipsyl crystals, such experiments are not easily carried out. 2) It is possible that histamine is more easily absorbed from the gastro-intestinal tract when present in complex with formaldehyde. The rapid onset of the toxic manifestations after the administration of histamine-formaldehyde supports the hypothesis of altered absorption. 3) It is possible that histamine when present in complex with formaldehyde is protected from inactivation in the gastro-intestinal tract and/or tissues subsequent to absorption. It has previously been shown that cyclic complexes of histamine are not degraded by histaminase (MONDOVI, SCIOSCIA-SANTORO ROTILIO & COSTA 1964). If protection from tissue inactivation is essential, it implies that less of the histamine-formaldehyde complex is present in the tissues when the two substances are given by different routes, than when they are both given by mouth. Assuming that the toxic manifestations observed in the present study are due to effects of free histamine, the substance must at some point be released from the complex with formaldehyde.

In addition to the above mentioned hypotheses more unlikely ones also exist. A further discussion of the various possibilities, however seems useless, until additional experimental evidence is available

Summary

When given orally to sheep in amounts of 0 02 mole or larger HCHO increased the fraction of simultaneously administered histamine which could be recovered as urinary free histamine. When 0.2 mole of HCHO and 0.3 g of histamine base were given, the urinary excretion of free histamine was about 35 times larger than when histamine alone was given moreover toxic manifestations were then invariably observed.

HCHO injected intravenously in amounts of 0.01 mole did not influence the fraction of orally administered histamine which was excreted unchanged in the urine. Hence inhibition of tissue inactivation of histamine by HCHO as demonstrated for sheep liver and kidney homogenates in the present study hardly explains the effect of HCHO on the urinary excretion of histamine when HCHO is given simultaneously with histamine by mouth

HCOOH given orally together with histamine in amounts of 0.2 mole or less did not affect the fraction excreted as urinary free histamine. Inhibition of tissue inactivation of histamine by HCOOH ($> 5 \times 10^{-3}$) was demonstrated.

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On the Clearance of Thorotrast ® a Dextrin Stabilized Colloid

By

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The rate at which the reticulo-endothelial system (RES) removes particles from the circulating blood is considered to consist of three phases represented by different slopes depending on the concentration of the substances being phagocytozed (MAXFIELD & MORTENSEN 1941). There is a concentration in which each particle in contact with a phagocytic cell can be expected to be taken up At a lower concentration a tail is found partly due to non-homogeneity in particle size and partly due to re-circulation of particles already taken up At higher concentrations the phagocytic cells are not able to take up all the particles which are offered for phagocytosis and an overloading phenomenon is present.

Thorotrast has been considered suitable for studies on phagocytosis because of the virtual absence of acute complications apart from a tendency to bleeding just after the injection.

The object of the present study was to investigate the behaviour of Thorotrast especially the clearance from the blood after i.v. injection into rabbits as evidence had been accumulating in previous studies (MÜLLER, unpublished), that higher doses of Thorotrast will cause another initial phase of clearance.

Materials and Methods

Thorotrast ® A colloidal solution of thori m dioxide, containing bout 25 % by volume of this compound, stabilized with 25 % tapioca dextrin, supplied by Fellows Testagar" Detroit, Mich.

Rabbits Young females weighing from 3 kg to 4.5 kg were used. If not stated otherwise, the injection of thorotrast was performed into the ea vein. I the experiments with prolonged injections and in which direct passage of the injected material through the liver is

wanted, the injections were given into the mesenteric vein with 0.5 ml Nembutal Sodium ® (pentobarbital sodium)/kg as basal anaesthesia. The anaesthesia was maintained by supplementary 0.5 ml Nembutal-Sodium every 45 minutes and supplemented by sufficient ether. Blood was used directly for analysis. Organs were removed as rapidly as possible and were homogenized in a Waring blendor diluted with saline to a known volume and samples taken for analysis. Before analysis of dextrin in the organs, th animals were fasted for 24 hours. All rabbits were sacrificed after one experiment.

Thorium analysis. The content of thorium (Th) or thorium dioxide (ThO_2) in the blood and organs was determined by the method of THOMASON *et al.* (1949) modified by MALTHIN (unpublished). Samples containing less than 1 mg of Th are digested with 5 ml concentrated nitric acid and then boiled with 2 ml concentrated sulphuric acid until the sample is colorless. After dilution the thorium is precipitated by addition of concentrated ammonium hydroxide to phenolphthalein red.

The overnight precipitate is dissolved in concentrated hydrochloric acid after washing and diluted to a fixed volume. An aliquot of this dilution gives a coloured complex with 1-(o-arsenophenylazo)-2-naphtol 3 6-disulfonic acid, sodium salt, in dilute hydrochloric acid. The colour obeys Beers law at density values below 0.600 d is measured at 545 mμ. Analytical error ± 1 ,.

Dextrin analysis. Samples (500 μl) are deproteinised by addition of 2.5 ml of 2 M perchloric acid. After w hing the precipitate with 1.0 ml of 2 M perchloric acid, the combined supernatants ar hydrolized without further addition of acid on a boiling waterbath for ½ hours. The samples are then diluted with distilled water to 10 0 ml and aliquots of 200 μl ar analyzed by the glucose determination method of Benedict ad. mod. FRANK *et al.* (1950). Analytical error ± 5 ,.

The colloidal ¹⁹⁸Au-preparation GCS.1P was obtained from The Radiochemical Centre, Amersham, England.

Heat denatured ¹³¹I-albumin was prepared accordi g the method of TAPLIN *et al.* (1961). The raw material was rabbit serum albumi to which was added the necessary amount of radioactivity i the form of human serum ¹³¹I-albumin received from Institut for Atomenergi, Kjeller Norway.

The radioactivity was measured in a Well-type scintillation crystal connected with pulse-heigh channel analyser.

The particle siz was determined by electronmicroscopy using a Siemens Elmiskop.

The dextrin was 25 / solutio of tapioca dextrin from the same batch as used for the preparation of an earlier supply f thorotrast from Heyden Chemical Corporation, Princeton, N.J.

Results

After injection of 3–4 ml Thor
clearance curve is obtained,
two easily distinguishable p
The first part of th (
an extremely low
in the blood in
phase lasts for
increase occurs
1–2½ hours.

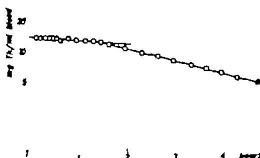


Fig. 1 Clearance of Thorotrast from blood after i.v. injection of 3.5 ml Thorotrast/kg rabbit. *Ordinate* mg Thorotrast/ml blood. *Abscissa* hours after injection.

The duration of the first phase is characterized by a very low rate of clearance and is dependent on the amount of Thorotrast injected (fig. 2). With a dose as low as 1 ml Thorotrast/kg the first bend seems to appear within 10 min after the injection.

The first phase could be due to a blocking of the reticulo-endothelial system by Thorotrast resulting in a lack of capacity to phagocytize Thorotrast as well as other colloids. Another possibility is that only Thorotrast could not be phagocytized because of a stabilizing factor in this colloid. In order to examine both possibilities experiments were performed to determine whether the presence of Thorotrast would prevent the disappearance of other colloids such as colloidal ^{198}Au and colloidal heat denatured ^{131}I-serum albumin. The Thorotrast and the marked colloid were injected simultaneously. Fig. 3a shows that the phagocytosis of the colloidal gold particles starts at a considerable rate immediately after the injection of the combined solutions which is also the case with colloidal gold in the absence of Thorotrast. Fig. 3b shows a similar result with colloidal heat denatured ^{131}I-serum albumin.

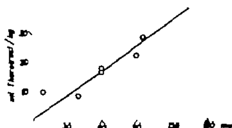


Fig. 2. Correlation between Thorotrast dosage and duration of the first phase of clearance.

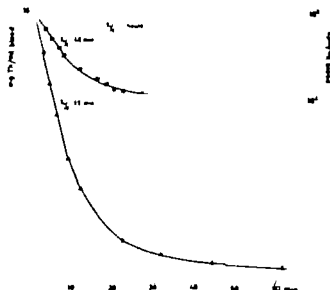


Fig. 3a. Clearance of colloids from blood after i.v. injection. Thorotrast ○ *2.4 ml/kg and* ^{198}Au □ (1.6 mg Au) injected simultaneous. ^{198}Au △ (9 mg Au) injected alone.

The distribution of thorium dioxide and of colloidal gold in the main depository organs can be seen in table 1. It is shown that practically all the colloidal gold is taken up by the liver during the first 30 min. contrary to this only a small percentage of Thorotrast is taken up by the liver and spleen during the first hour. When the rate of Thorotrast uptake increases, the distribution between the liver and spleen is different with close to a fifth of the injected amount being deposited in the spleen.

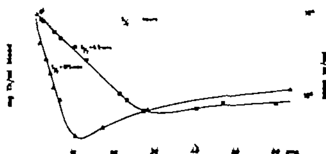


Fig. 3b. Clearance of colloids from blood after i.v. injection. Thorotrast ○ (2.7 ml/kg) and heat denatured ^{131}I-serum albumin □ (10 mg) injected simultaneously. Heat denatured ^{131}I-serum albumin △ (5 mg) injected alone.

Table 1

Distribution of colloidal gold and of thorium dioxide in the main organs f the RES.

Inj. dose pr kg	Rabbit no.	Colloid	Time after injection	Per cent of total amount injected		
				liver	spleen	lungs
10 mg Au	1	198A	31 min.	98.9	0.08	0 04
9 mg Au	2	198Au	5 days	99 9	0 1	0 1
598 mg ThO_2	3	Thorotrast	60 min.	16.2	0 4	
852 mg ThO_2	4	Thorotrast	3 days	56.9	11.3	2 48

Particle size could be an explanation for the difference in the handling of these colloids in comparison to Thorotrast, as suggested amongst others by SMULDERS (1951) However this appears improbable as the particle size of the radioactive gold colloid was found to be between 1–5 nm which is of the same order of magnitude as thorium dioxide particles in Thorotrast, where the particle size is 2–10 nm The particle size of the denatured serum albumin which was also determined by electronmicroscopy was found to vary between 20–200 nm The experiment suggested that Thorotrast is kept circulating in the blood without any immediate uptake into the reticulo-endothelial system. It therefore seemed of interest to study the behaviour of Thorotrast after continuous intravenous injection. Fig. 4 contains the results of 4 such experiments. The injection was performed continously into a mesenteric vein at a



Fig. 4. Correlation between Th-concentration in blood and injected volume of Thorotrast during continuous injection at different speed in the mesenteric vein. ◇ = 0.8 ml/min. O = 0 4 ml/min. □ = 0.2 ml/min. Δ = 0.1 ml/min.

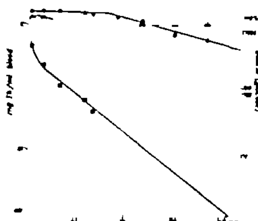


Fig. 5 Th (○) dextrin (□), and glucose (△) concentrations i blood after i.v injection of 3.5 ml Thorotrast/kg.

constant speed by means of an injection machine. In the four experiments the speed varied from 0.8 ml/min. to 0.1 ml/min. It is seen that during these injections lasting from 15 min. to 120 min. the thorium concentration in the blood is directly proportional to the amount injected at each rate of injection and furthermore that the colloid can not be phagocytized during the first passage through the liver

As mentioned above Thorotrast contains both thorium dioxide and a relatively large amount of dextrin. Fig. 5 shows the changes in dextrin concentration in the blood with time after an injection of Thorotrast. Within 30 minutes more than 90 / of the dextrin has been removed from

Table 2

Th/dextrin ratio in blood at the bend point after different doses of Thorotrast.

Exp. no	Dose ml Th/kg	Bend point min.	mg % Th	mg – dextrin	Th/ dextrin	Mean
1	3 5	78	1430	48	29 7	
2		75	1270	71	17.9	20.6[1])
3	–	73	1440	102	14.2	
4	1 75	45	898	70	12.8	13 1[1])
5		65	618	47	13.2	

[1]) No difference between the means (P > 0.3 by t-test).

the circulating blood. Obviously this is not entirely due to enzymatic reactions, since much of it can initially be found in the liver without the corresponding amount of Th-dioxide The blood sugar concentration in the serum increases shortly after the injection and the highest values are reached between 5 and 20 minutes later and are 50 to 75 mg / above the fasting level. The blood sugar of the rabbit is not a very stable value. It could, however be shown that under the same conditions the injection of India ink is without influence on the blood sugar level

Experiments have been carried out with different doses of Thorotrast in order to obtain an estimate of the relative amounts of dextrin and thorium present in the circulating blood, at the time of the bend. The results are shown in table 2.

Discussion

That Thorotrast injected intravenously will stay in the circulation for some time, if sufficiently large doses are used, as shown chemically in these experiments, has also been observed by EKHOLM *et al* (1964). They report that high doses of Thorotrast injected intravenously into cats remain in the circulation for 30 min. up to 1 hour and permit X-ray studies of the vascular system without the introduction of fresh contrast media.

The only thorough study of the disappearance of Thorotrast down to low concentrations after injection of different doses in rabbits, was published by MAXFIELD & MORTENSEN (1941) who used the radioactivity of thorium to measure the concentration. They found initially a slow rate of clearance. After an abrupt transition the rate of phagocytosis became much faster and finally after a second change in the clearance rate, a third phase set in with a very slow disappearance rate. The highest dose of Thorotrast used was 1 ml/kg bodyweight, but even then the initial rate of clearance is of quite a different order of magnitude than the one found in this study This makes it probable that their first stage might not be identical with the first stage demonstrated in the present study The Th-concentrations calculated to be present in the two experiments support this difference

The mean Th-concentration at the first bend of MAXFIELD & MORTENSEN (1941) can be calculated to be 0.75 mg Th/ml blood, assuming that the rabbits had an average weight of 3.5 kg with a blood volume of about 45 ml/kg. In the present work the Thorotrast dose used is 3.5 ml/kg which gives a Th-concentration at the first bend of 11 mg/ml According to MAXFIELD & MORTENSEN (1941) their first phase was due to overloading. If the bend described in the present paper represents the lower limit of phase with overloading, the concentration of Th at the followi

should be of the same order of magnitude as the one found by MAXFIELD & MORTENSEN (1941) Furthermore it can be seen from fig. 2 that both the time of the bend and the Th-concentration at this point are dose dependent. This would suggest that it is the second phase of the Th-clearance curve in the present study which is the one identical with MAXFIELD & MORTENSEN's (1941) period of overloading. A comparison of the slopes of the second phase in the present experiments and the first phase published by MAXFIELD & MORTENSEN (1941) shows half life values of the same order of magnitude, at 1–2 hours. Furthermore the Th-concentration at the second bend in the present study and at the first bend in the study by MAXFIELD & MORTENSEN (1941) is about 0 8 mg Th/ml blood which makes the named phases identical in both studies That MAXFIELD & MORTENSEN (1941) did not find this initially slow fall in Th-concentration is probably due to the fact that the first Th-determination was taken too late. A study of their clearance curve after the highest dose injected could in view of our present knowledge, suggest that such an initially slower fall in concentration was actually present.

No change in the Th-concentration could be demonstrated within the first 70 to 90 minutes after the injection of larger amounts of Thorotrast. Further the clearance of iodinated rabbit serum albumin and colloidal gold is practically unchanged in the presence of Thorotrast. This shows that no blocking of the reticulo-endothelial system occurs by Thorotrast against colloids These facts may therefore explain why Thorotrast was kept circulating in the blood in order to be treated in such a way as to make ingestion by phagocytes possible Support for this possibility can be obtained in various ways.

Thorium dioxide apart from the hydrophilic colloid Thorotrast can exist as a hydrophobic colloid which is used as a precursor in the manufacturing of Thorotrast. This colloid contains no protective substance and precipitates readily with small concentrations of double or triple charged negative ions of inorganic electrolytes and with proteins. However the thorium dioxide in Thorotrast is protected by dextrin, the removal of which might cause a slow conversion against the hydrophobic state Fig. 5 shows that while the Th-concentration is practically unchanged during the first 1½ hours after injection while concentration of dextrin in serum decreases to about 2 / of the initial value. This suggests that there is a threshold value for the amount of dextrin which has to be present in order to prevent Thorotrast from being bound to proteins in the plasma. Such a binding was demonstrated by BENNHOLD (1938) who showed in kataphoresis, that Thorotrast was bound quantitatively to globulins in a concentration of 2.2 ml Thorotrast/53 ml serum, but further details are unfortunately not available. As dextrin is the only stabilizing agent in the hydrophilic

colloid Thorotrast, it is also assumed to prevent the Th-dioxide particles in the blood from being coated with protein as a first step to being ingested by the phagocytes. The Th/dextrin ratio at the bend point has been determined with different doses of Thorotrast (table 2)

The ratios in the two experiments show no significant difference. Therefore there may be a threshold value for the amount of dextrin needed to prevent phagocytosis of Thorotrast.

The role of dextrin as a protecting factor against phagocytosis fits in well with our observation that Thorotrast practically does not affect the simultaneous clearance of a gelatin stabilized gold colloid and a heat denatured albumin colloid Furthermore Wood (1960) has stressed that bacteria with a polysaccharid containing capsule are phagocytized with greater difficulty than bacteria without this coating.

Summary

The initial part of the clearance curve of Thorotrast ® after i.v injection of doses between 1 and 3.5 ml/kg into rabbits has been studied. An unexpected primary phase with an extremely low rate of clearance has been demonstrated with a duration dependent on the amount of Thorotrast injected No blocking of the reticulo-endothelial system by Thorotrast could be demonstrated Most of the dextrin is removed soon after injection without the corresponding amount of thorium dioxide As dextrin is the only stabilizing agent in Thorotrast, there seems to be a threshold value for dextrin in the blood which protects the colloid from being phagocytized

Acknowledgements

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Release of α-Methylated Noradrenaline Analogues by Nialamide

By

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(Received August 14, 1968)

In a previous investigation we found that inhibitors of the monoamine oxidase (MAO), such as nialamide, are capable of releasing metaraminol (MA) taken up in the adrenergic neurone (CARLSSON & WALDECK 1966). This release, which is also effective on erythro α-methylnoradrenaline (α-Me-NA), has been shown to be mediated by monoamines, i.a. noradrenaline (NA) which accumulates as a consequence of the MAO inhibition (CARLSSON, LINDQVIST & WALDECK 1968).

In the present investigation we have further analyzed the effect of nialamide on ^{3}H-metaraminol and erythro ^{3}H-α-Me-NA in the mouse heart. We have also included in our study the threo isomer of the last mentioned amine.

Material and Methods

Female mice were used in all experiments. The methods used for tissue extraction and the estimation of labelled amines have been described elsewhere (CARLSSON, LUNDBORG, STITZEL & WALDECK 1967, CARLSSON & WALDECK 1965, WALDECK 1968). Erythro and threo ^{3}H-α-Me-NA and ^{3}H-metaraminol were prepared in cooperation with the Research Laboratories of Hässle Ltd., Göteborg (HALLHAGEN & WALDECK 1968). Other experimental details are given under Results.

Results

Nialamide was given i.p. in a dose of 100 mg/kg 2 hr before the i.v. administration of erythro-^{3}H-α-Me-NA. The animals were killed at various time intervals after the administration of the labelled amine. Controls received ^{3}H-α-Me-NA only. The disappearance of the amine from the hearts of the controls appeared to take a biphasic course, being

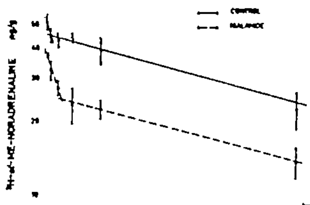


Fig. 1 Effect of nialamide on erythro ^{3}H-α-methylnoradrenaline in the mouse heart. Nialamide, 100 mg/kg i.p was given to mice hr before the i v administration of 0.02 mg/kg erythro ^{3}H-α-methylnoradrenaline. At various time intervals after the labelled amine the animals were sacrificed. Controls received erythro ^{3}H-α methylnoradrenaline only The data re the means ± S. E.M of in general 5 experimental groups, each group consisting of 6 animals. One of the control values at 2 hr which was extremely high (70 ng/g) possibly due to contamination, wa not included in the mean.

more rapid during the first hour The initial uptake was only slightly reduced by nialamide (fig. 1) During the first 1–2 hr after the injection ^{3}H-α-Me NA disappeared much more rapidly in the nialamide pretreated animals than in the controls. After this, however no difference in the rate of turn-over was observed

For comparative purposes data from a previous investigation on ^{3}H MA (Carlsson & Waldeck 1966) are presented in fig. 2. Here the disappear

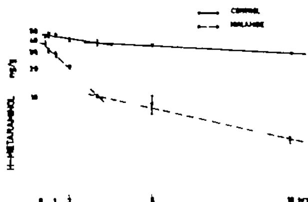


Fig. 2. Effect of nialamide on ^{3}H-metaraminol in the mouse heart. The experimental conditions are analogous to those described in Fig. 1 (From Carlsson & Waldeck 1966).

Table 1

Effect of nialamide on ^{3}H-metaraminol, erythro and threo ^{3}H-α-methylnoradrenaline i the mouse heart. Mice were given nialamide, 100 mg/kg i p., 2 h before the i.v administration of either f the labelled amines. The animals were killed t various time intervals thereafter The means ± S.E.M. are shown. Figures in brackets denote the number of experimental groups, each group consisting of 6 animals.

Labelled amine 0.02 mg/kg	Time interval hr	Control g/g	Nialamide ng/g	per cent of control
	2	43 ± 2 (6)	22 ± 1 (4)	51
^{3}H-metaraminol[1])	4	40 ± 3 (6)	11 ± 1 (4)	28
	18	32 ± 1 (5)	3.8 ± 0.3 (2)	12
Erythro ^{3}H-α-methyl-noradrenaline	2	45 ± 2 (5)	24 ± 4 (5)	53
	4	40 ± 5 (5)	23 ± 2 (5)	57
	18	23 ± 4 (5)	14 ± 2 (5)	61
Threo ^{3}H-α methyl-noradrenaline	2	31 ± 1 (3)	12 ± 1 (3)	39
	4	34 (1)	8.3 (1)	24
	18	15 ± 1 (4)	1.3 ± 0.5 (4)	9

[1]) Data from CARLSSON & WALDECK (1966)

ance of the test amine was also biphasic. In contrast to ^{3}H-α Me NA a releasing effect of nialamide was also demonstrated after the first 2 hr

In another experiment the amine-releasing effect of nialamide was tested on threo-^{3}H-α Me NA. The experiment was performed as described above except that only the time intervals 2, 4 and 18 hr were included

Table 2

Effect f nialamide on H-metaraminol and erythro H-α-methylnoradrenal ne in the mouse heart 3 days after administration of the amine. ^{3}H Metaraminol or erythro ^{3}H-α methylnoradrenaline (^{3}H-α Me-NA), was given to mice Three days later nialamide, 100 mg/kg, was given p. and the animals were killed after another 4 hr Controls received the labelled mine only The means ± S.E M f 7 (^{3}H-metaraminol) and 8 (^{3}H-α Me NA) experimental groups are shown, each group consisting of 6 mals.

Labelled amine 0.02 mg/kg	Control ng/g	Nialamide g/g	Nialamide per cent of control
^{3}H-metaraminol	20.7 ± 1 6	8.0 ± 0.6	39
^{3}H-α-Me-NA	11.5 ± 1 4	10.9 ± 1 4	95

In table 1 the 2, 4 and 18 hr values from all the three series of experiments were collected In addition to the absolute amine levels the data have been calculated as per cent of the respective control values. It appears that the nialamide-induced release pattern of threo-^{3}H-α Me NA is more like that of ^{3}H MA than that of erythro-^{3}H-α-Me NA in that the release continues after 2 hr

Furthermore, ^{3}H MA or erythro-^{3}H-α Me NA was given to mice and 3 days later some of the animals received nialamide, 100 mg/kg i.p After another 4 hr the animals were killed and the respective labelled amines in their hearts determined The results in absolute amine levels and in per cent of respective control values are shown in table 2. The data show that 3 days after administration ^{3}H MA can still be released by nialamide whereas erythro-^{3}H-α Me-NA appears to be uninfluenced under these conditions. Attempts were also made to study the effect of nialamide on threo-^{3}H-α Me NA in the same way but after 3 days this amine had reached too low values to make significant measurements possible.

Discussion

The amine releasing action of nialamide has been shown to be mediated by endogenous monoamines, *i.e* NA, accumulating as a result of MAO inhibition. The present investigation has revealed a difference in the effect of nialamide on erythro-α-Me-NA and MA (fig. 1 and 2). Whereas the rate of disappearance of MA was increased by the drug during the whole period of observation (18 hr) the effect on erythro-α Me NA ceased after one hour at which time it had reached half the control value. The threo form of α-Me-NA appeared to behave more like MA than its diastereoisomer (table 1) The interpretation of these data may be that the different amines and their isomers have different affinities for the amine-storage sites of the adrenergic neurone (+)-MA has been shown to be briefly taken up by the heart (SHORE *et al* 1964). Since the ^{3}H MA used in the present study was racemic it is possible that the dextrorotatory isomer is in part responsible for the first rapid disappearance of MA.

Accordingly (+)-erythro-α-Me NA would be released initially by the effect of nialamide. The remaining ( )-isomer of erythro-α Me-NA would thus be uninfluenced by nialamide since after 2 hr there was no difference in the rate of disappearance between the control and the nialamide pretreated groups (fig. 1) Further support for this view is given by the finding that 3 days after administration erythro-^{3}H-α-Me NA was uninfluenced by nialamide (table 2) Moreover α-Me NA formed from α-Me-DA

In vivo (probably ( )-erythro-α Me NA) appears to be insensitive to the releasing action of nialamide (CARLSSON, MEISCH & WALDECK 1968).

Pretreatment with an inhibitor of the MAO causes a decreased rate of disappearance of ³H NA (AXELROD *et al* 1961) This apparent paradox in the action of MAO-inhibitors on the α methylated amines and NA may be explained by the inhibition of the normally occurring intraneuronal destruction of NA by the MAO Since the nialamide-induced amine release is prevented by inhibitors of dopa-decarboxylase (CARLSSON LINDQVIST & WALDECK 1968) it might be suggested that inhibition of this enzyme in addition to the MAO would cause a further decrease in the rate of disappearance of ³H NA. Attempts to show such an effect have not so far been successful (unpublished data)

It thus appears that NA and its analogues differ in sensitivity to the amine-releasing action of nialamide, MA and threo α Me-NA being more sensitive than erythro α Me NA. In fact, it is doubtful whether ( )-erythro α Me NA and NA are at all affected by this releasing action. These differences probably reflect different affinities of the amines for the reserpine-sensitive storage mechanism of the amine granules.

Summary

Tritium labelled erythro or threo α-methylnoradrenaline or metaraminol was given intravenously to mice. The effect of the monoamine oxidase inhibitor nialamide, on the uptake and retention of these amines in the heart was investigated. It was found that threo ³H-α methyl-noradrenaline was more efficiently released by nialamide than was the erythro form In this respect it was more like ³H metaraminol. Three days after the administration of the labelled amines, nialamide has no effect on erythro ³H-α-methylnoradrenaline whereas ³H-metaraminol could still be released The data indicate *different affinities of the amines and their stereoisomers* for the reserpine-sensitive storage mechanism of the amine granules

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Distribution and Metabolism of 3,3-dimethyl-1-(3-methylaminopropyl)-1 phenyl-phthalane (Lu 3-010), a Bicyclic Compound with Thymoleptic Properties

By

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(Received April 27 1968)

In a previous publication (PETERSEN *et al* 1966) the chemical and pharmacological properties of a series of new bicyclic compounds were described. Among these the phthalanes were found to be the most potent and of these Lu 3-010 (formula see fig 1) was chosen for further studies. The purpose of the present publication is to describe the distribution and metabolism of this compound in detail.

Materials and Methods

A. *Labelling*

The labelled substances used in experiments were *Lu 3-010-³H(G)* made by WILZBACH labelling (exposure of the finely powdered HCl-salt to ~1C tritium-gas), repeated dissolution in ethanol and evaporation to dryness, repeated alternating extraction of base and HCl-salt in water-ether system and precipitation of HCl-salt from dry acetone solution.

Control of purity was made by thin layer chromatography (TLC). Different exposure times gave different specific activities

I	11 days	app. 3 μC/mg
II	21 days	app. 17 μC/mg
III	6 weeks	app. [illegible]6 μC/mg

Mainly subst. II was used in the experiments. Yield after purification was 50–60 % of batch of 300 mg. Radiochemical purity was > 90 %. *Lu 3-010-¹⁴C* made by synthesis, the label situated in the methylene group alpha to aminonitrogen. Recrystallization was made from ethanol-acetone mixture (as HCl-salt) and the substance controlled as mentioned above.

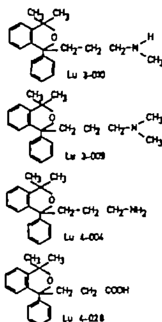


Fig. 1 Reference substances.

Specific activity was 9 µC/mg.
Radiochemical purity was > 95%.
Melting points were in the range [188–91
pK_A = 9.3
The radiochemical impurities followed the substance in the solvents used for extraction.

B. *Isotope technique*

All measurements were made in Packard Tri-carb liquid scintillation spectrometer model 3314 with automatic external standardisation. The scintillation medium used was the dioxane-methanol-toluene-naphthalene system (diotol) described by Herberg (1960) modified by replacing POPOP by dimethyl-POPOP

Blood. To 0 1–0.2 ml blood samples were added equal volumes of 30% hydrogen peroxide and 1 ml 0.5 M ethanolic potassium hydroxide. After incubation at 55 for 3 hours 10 ml [diotol solution were added, and the samples were then ready for counting.

Urine, bile To 0.1–0.5 ml urine or bile samples were added 10 ml of diotol solution and the samples were counted.

Tissues, faeces. From the rat tissues 10 / water homogenates were made and 0.5 ml samples were counted as the urine samples. Dog tissues were homogenized in 0.5 M ethanolic potassium hydroxyde and incubated *overnight* t 55 Of these homogenates 1.0 ml samples, corresponding to 100 mg wet tissue, were counted in 10 ml diotol. All faeces samples were handled like dog tissue. The rat carcasses were ground and handled like dog tissue, except that the homogenates were boiled under reflux for 8 hours.

Autoradiography Mice (20–25 g) were given 20 mg/kg i.v [*Lu 3-010-*^{14}*C.* After 20 or 60 minutes the animals were anaesthetized with ether and killed by immersion in heptane

cooled with solid carbon dioxide. Whole body autoradiography was performed as described by ULLBERG (1958), using 40 μ section and Structurix D-7 X ray films (Agfa Gevaert). Exposure time was 3 weeks.

C. Chromatography

TLC was carried out on glassplates (20 × 20 cm) coated with a 250 μ layer of Silica Gel G according to STAHL (MERCK) and activated at 110° for 30 minutes.

The following solvent systems (all unsaturated) were used

1. cyclohexane acetone diethylamine 50 30 5
2. chloroform acetone diethylamine 30 70 0.5
3. cyclohexane acetone acetic acid 80 20 1
4. benzene acetic acid 95 5
5. methanol water 90 10.

For the detection of spots the following spray reagents according to STAHL were used

1. sulphuric acid, conc. (UV-light 366 nm)
2. ninhydrine (amines)
3. sodiumnitroprusside-acetaldehyde (sec. amines)
4. bromcresolgreen (acids)
5. fast blue salt B (phenols)
6. ferrichloride-potassiumferricyanide (phenols)

As reference substances Lu 3-010 and some analogous substances named Lu 3-009 Lu 4-004 and Lu 4-028 were used (for formulas see fig. 1).

After chromatography of radioactive extracts the silicagel was divided into 1 × 1 cm squares. Each square was scraped off into a counting vial and counted in 10 ml of ditol.

D. Extraction procedures

Urine, faeces- or organ-homogenates were extracted at pH = 1 and pH = 9 or pH = 13 by shaking three times with double the volume of cyclohexane for 20 minutes. Separation was achieved by centrifugation. For TLC, the extracts were evaporated and the dry residue resolved in chloroform. β-Glucuronidase or hydrochloric acid hydrolysis was performed on the urine or organ-homogenate after extraction.

E. Excretion and distribution in rat and dog

Rat studies. The distribution study was performed with 22 male Sprague-Dawley rats, 76–87 g, fasted for 24 hours. The animals were given 5 mg *Lu 3-010-3H/kg* intravenously corresponding to 83 μC/kg. At different intervals 2 animals were anaesthetized with ether and exsanguinated. The organs were then removed and the isotope content determined. The isotope content of the carcass was determined. The time intervals were 5, 15, 30 min., 1, 2, 4, 8 hours and 1, 2, 4, 8 days after injection. The excretion study was performed with 6 male Sprague-Dawley rats, 99–106 g, fasted for 24 hours. The animals were given the same dose as in the distribution study plus 1 ml water orally. The animals were kept in separate metabolic cages with water and food ad libitum. The urine and faeces were collected at 1, 2, 4, 8, 12, 24 hours and thereafter every day for 8 days. At the end of the experiment the animals were sacrificed with ether, ground and the remaining activity determined.

Dog studies. Identification of metabolites was performed on urine collected from dogs given daily dose f 25 mg/kg orally for 6 months.

The distribution study was performed on mongrel male dogs, weighing 11.4 nd 16.8 kg respectively

The dogs were given 1 mg *Lu 3-010-*^{14}*C/kg* intra enously corresponding to 9 μC^{14}C/kg. After 1 hour the dogs were sacrificed with 30 ml 6.0 mebumal-sodium given intravenously The organs were removed and the activity determined

Excretion studies Two male beagle dogs were anaesthetized with thiopentone sodium dministered intr venously A bile fistula was made according t the method of Rous & Mc Master (1923), and the dogs were allowed to recover for 4 days. Both dogs ere treated with the bov mentioned dose of *Lu 3-010-*^{14}*C* intravenously After 15 min., 1 4 8, 4 48 and 7? hours, blood urine and bile samples were collected. Faeces samples were collected 36 nd 48 hours fter the injection. At the end of the experiment both dogs were sacrificed with mebumal-sodium given intravenously

A supplementary experiment was carried out on t female beagle dogs. The dogs were anaesthetized with thiopentone sodium given intravenously and the narcosis was prolonged by halothane inhalation. A bile fistula was made according to the method mentioned above. A permanent catheter was installed in the urinary bladder A permanent polythene catheter wa inserted in the jugular vein and the dogs were given 1 mg/kg of *Lu 3-010-*^{1} *C* intravenously U der halothane narcosis urine and bile samples were collected quantitatively for the following periods 0–15, 15–30 30–60 60–120 120–240, 240–360 and 360–480 minutes. The dogs were sacrificed with 6 mebumal-sodium given intravenously and the material from the liver gallbladder salivary gland and pancreas collected and tested for their isotope content. The radioacti ity of the gastrointestinal contents was measured.

F *N-demethylation in vitro*

I vitro N-demethylation rates were determined on 9000 g supernatant from rat liver homogenate as described by Bickel et al. (1967)

G *Inhibition of monoamineoxidase* (MAO)

M le White rats (100–120 g) were pretreated for three days with 10 mg/kg per day of β-p-chlorphenylmercapto-ethylhydrazine, very potent MAO-inhibitor described by Møller Nielsen & Huus (published (1960)[1]). This dose gives almost complete inhibition f MAO On the fourth d y 50 mg/kg f *Lu 3-010* or Lu 4-004 was given orally in addition to the MAO-inhibitor Urine was collected for the following 24 hours and extracted for chromatography

Results

Identification of metabolites

In the chromatographic studies with unlabelled compound the parent substance and two metabolites were found These were identified by comparison with reference substances (R_f-values are shown in table 1).

[1]) Paper read t the Joint meeting of the British and Scandinavian Pharmacological Societies, Copenhagen, July 1960.

Table 1

R_f-values of reference substances.

	System 1	System 2	System 3	System 4	System 5
Lu 3-010	0.46	0 12	0	0	0 08
Lu 4-004	0.72	0.41	0	0	0 11
Lu 3-009	0.85	0.25	0	0.01	0.15
Lu 4-028	0	0	0.35	0.47	0 80

One of the metabolites was extracted at pH = 9 or pH = 13 and showed R_f-values and colour reactions with the spray reagents identical to the primary amine Lu 4-004 The other metabolite was extracted at pH = 1 Its R_f-values and colour reactions were identical to the acid Lu 4-028 The two metabolites were thus considered to be Lu 4-004 and Lu 4-028

Lu 4-028 was completely extracted only after acid hydrolysis indicating that this compound was present to some extent in a conjugated form in the urine. However it cannot be a glucuronic acid conjugate since Lu 4-028 was not liberated by incubation with β-glucuronidase It may well be an unspecific binding.

The metabolites suggest the metabolic pathway presented in fig. 2.

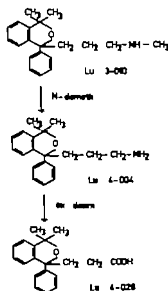


Fig. 2. Metabolic pathway of Lu 3-010.

Table 2

N-demethylation rates.

	% demethylated in an hour
Lu 3-010	
Nortriptyline	7
Desipramine	7
Amitriptyline	44
Imipramine	44

The N-demethylation rates in vitro of *Lu 3-010* and some other compounds are shown in table 2. The N-demethylation of Lu 3-010 was found to be a slow process as compared to the N-demethylation of nortriptyline and desipramine which in turn are very slow processes as compared to the N-demethylation of amitriptyline and imipramine.

The second step in the degradation is believed to be catalyzed by the enzyme monoamineoxidase (MAO), since pretreatment of rats with a MAO-inhibitor considerably diminished the excretion of Lu 4-028 following administration of *Lu 3-010* or Lu 4-004. The excretion of Lu 4-004 following administration of *Lu 3-010* was not perceptibly changed.

Distribution and excretion in rats

The distribution of tritium after intravenous injection of *Lu 3-010-*^3H is shown in table 3. The highest amounts of radioactivity were found in the liver and the lungs reaching a maximum between 30 and 60 minutes. The isotope concentration in the brain was low compared to the concentration in the liver and the lungs but of about the same order as the concentration in the fat and the blood. The highest total amount of radioactivity found in the stomach and intestines, i.e. 25 % of the administered radioactivity was found 2 hours after injection. Tissue homogenates from the brain, liver and lungs were extracted at pH = 13 followed by extraction at pH = 1. The amounts of radioactivity excreted at the two pH s are given in table 4. Identification of the parent drug and formed metabolites in all cases was based on TLC in several different solvent systems, as described in materials and methods. In the alkaline extract of rat brain only two labelled compounds were found and identified as the parent drug and Lu 4-004, the primary amine. Fig. 3 shows the chromatogram of brain extract 15 min after injection. From the isotope data from the chromato-

Table 3

Distribution of total radioactivity following intravenously injected Lu 3-010-³H in rats (n = 2).

Organ	radioactive dose/g wet tissue				
	30 min.	60 min.	8 hr	24 hr	192 hr
Abdominal Fat	0.28	0.32	0 19	0.03	0.01
Blood	0.20	0.20	0.13	0.09	<0.001
Brain	0.22	0.25	0.19	0.06	0.02
Gastrointestinal tract	1.0	1.3	1 1	0.30	0.05
Heart.	1.3	1 3	0 6	0.09	0.03
Kidneys	2.8	2.6	1.3	0 15	0.04
Liver	3 6	2.3	1 7	0.44	0.05
Lungs	6.3	8 1	3.1	0.29	0.04
Spleen	2.7	2.9	1 9	0.17	0.07

grams, the ratio Lu 3-010 Lu 4-004 was calculated to 1 3 after 15 minutes and 1 5 after 8 hours The low amount of radioactivity in the acid brain extract did not allow any analysis. The alkaline extracts of the liver and lungs after various periods contained the parent drug and Lu 4-004 as the dominating compounds these being in roughly the same amounts. One or two not identified radioactive compounds were found in small amounts in most extracts. The acid extracts did not give much information but the occurence of Lu 4-028 was demonstrated by chromatography

The results from the excretion studies are illustrated in fig. 4 The excretion of the labelled drug and its metabolites is almost complete in 48 hours while 80 / is excreted during the first 24 hours. Of the adminster

Table 4

Extraction of rat tissues. Values given are per cent f total tissue radioactivity

Time after injection	Brain: Ex tracted at pH 13	Brain: Ex tracted at pH 1	Time after injection	Liver: Ex tracted at pH 13	Liver: Ex tracted t pH 1	Lungs: Ex tracted t pH 13	Lungs: Ex tracted at pH 1
0–15 min..	45	7	0–5 min.	47	1	98	2
15 min. 1 h	54	12	0.5–1 hr	70	3	75	1
2–8 hr..	34	11	8–24 hr	37	19	68	1

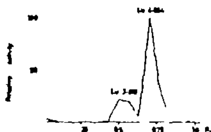


Fig. 3 Distribution of radioactivity in alkaline extract from rat brain 15 minutes after injection. TLC in solvent system 1

ed dose 45 / (range 38–50 %) was obtained in the urine, 44 / (37–52 /) in the faeces in 6 days while 2 / (1 4–2.5 /) remained in the carcass at the end of this period Maximum excretion rate in the urine, i e. 7 /hr., was obtained during the first hour after injection and in the faeces, 3 /hr was obtained 8–12 hours after injection The amount of radioactivity extractable at pH = 13 and 9 from urine decreases with time from 39 / during the first hour to 15 / during 24–48 hr The radioactivity in the acid extract increases from 2 / during the first hour to 34 / during 24–48 hours. In the faeces collected during the first 4 hours 94 / of the radioactivity was extracted at pH = 13 and 9 and this decreased to 14 / in the faeces collected between 24 and 48 hours. Corresponding values for acid extraction of the faeces were 5 / and 73 / respectively Chromatographic analysis of the urine extracts showed that in the alkaline extracts the parent drug and the primary amine Lu 4-004 were the only important compounds present as shown in fig. 5 In the first hour urine sample only the parent drug was found. In the acid extracts the acid metabolite Lu 4-028 was found to be the major compound. In extracts from the faeces the same compounds were identified as in the extracts from urine

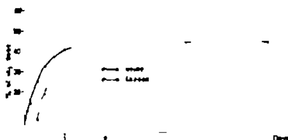


Fig. 4 Excretion of total radioactivity following intravenously injected Lu 3-010-³H in rats (= 6).

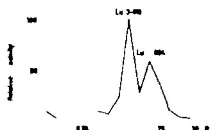


Fig 5. Distribution of radioactivity in alkaline extract from rat urine 4–8 hours after injection. TLC in solvent system 1

Distribution and excretion in dogs

The distribution of ^{14}C one hour after the intravenous injection of *Lu 3-010-^{14}C* in dogs is shown in table 5 As in the rats the highest concentrations are found in the lungs and in the liver The lowest concentrations are found in the blood, the fat and the brain. The drug is uniformly

Table 5

Distribution of total radioactivity following intravenously injected Lu 3-010-^{14}C in dogs (— 2)

Organ	% radioactive dose/g wet tissue	
	1 hr	72 hr
Abdominal Fat	3.4×10^{-3}	
Blood.	3.5×10^{-3}	
Bone marrow	$1.4 \times 10^{-}$	
Duodenum	1.8×10^{-2}	1.5×10^{-3}
Heart	$2.4 \times 10^{-}$	–
Ileum	1.4×10^{-2}	$2.3 \times 10^{-}$
Jejunum	$2.8 \times 10^{-}$	2.1×10^{-3}
Kidneys	$2.6 \times 10^{-}$	–
Liver	5.2×10^{-2}	5.5×10^{-3}
Lungs	7.3×10^{-2}	–
Pancreas.	$3.5 \times 10^{-}$	1.2×10^{-3}
Salivary gland	$2.1 \times 10^{-}$	1.9×10^{-3}
Spleen	$2.3 \times 10^{-}$	–
Brain Basal ganglia	$3.7 \times 10^{-}$	–
Brain Cerebellum	$3.9 \times 10^{-}$	–
Brain Cortex.	3.7×10^{-3}	–
Brain Medulla Oblongata	3.7×10^{-3}	–
Cerebrospinal fluid	1.3×10^{-4}	–

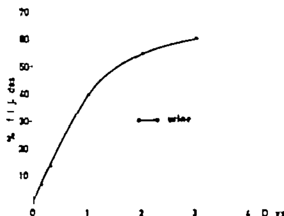


Fig. 6. Urinary excretion of total radioactivity following intravenously injected Ln 3-016-^{14}C in dogs (n = 2)

distributed in the brain as shown in table 5 Analysis of extracts from the liver and lungs showed the same metabolites as in the rat study and in both tissues the parent drug was dominant. Due to the low concentration of radioactivity in the brain identification of metabolites was very difficult and the only compound that could be clearly identified was the parent drug.

In the elimination study 94 / of the administered ^{14}C-dose was accounted for As shown in fig. 6 60 / was found in the urine, 40 / during the first 24 hours. Maximum excretion rate, i.e. 8 /hr., was found 15–60 min. after injection. In the faeces, 30 / of the dose was found in 72 hours and 24 / during 24–48 hours. No faeces sample was obtained during the first 24 hours. At the time of sacrifice 4 / of the dose was found in the colon. No activity was found in the bile at any time interval. Because of the total absence of radioactivity in the bile the second supplementary study was made, as described in methods. This study confirmed the result obtained in the first experiment At the end of the second experiment 6 / of the radioactive dose was found in the gastrointestinal tract. In the urine from

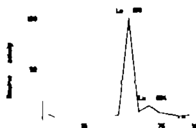


Fig. 7 Distribution of radioactivity in alkaline extract from dog rine 0–15 minutes after I jection TLC I solvent system 1

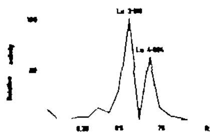


Fig. 8. Distribution of radioactivity in alkaline extract from dog urine 24–48 hours after injection. TLC in solvent system 1

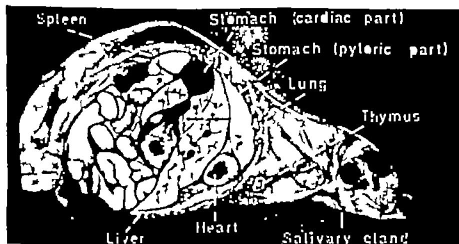


Fig. 9 Distribution of radioactivity (dark areas) 20 minutes after injection.

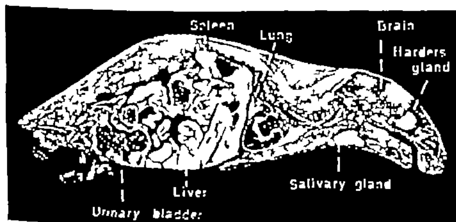


Fig. 10. Distribution of radioactivity (dark areas) 60 minutes after injection.

the first 15 min. 53 % of the radioactivity was extracted at pH = 13, but only 1 % in the urine after 72 hours. The acid extract increased from 12 to 64 % during the same period In the faeces collected during 24–48 hours 17 % of the radioactivity was extracted at pH = 13 and 35 % at pH 1 During the period 48–72 hours after the injection, 8 % of the radioactivity was extracted at pH = 13 and 58 % at pH = 1 As shown in fig. 7 and 8 the isotope content of the alkaline urine extracts is distributed between two compounds *Lu 3-010* and Lu 4-004 The entire isotope content in the acid extracts was identified as Lu 4-028 The analysis of extracts from faeces showed the same products as found in the urine.

Autoradiography in mice

The distribution of radioactivity after whole body autoradiography is presented in fig. 9 and 10

Discussion

As shown in fig. 2 the degradation of *Lu 3-010* is completely different from that of other thymoleptic drugs e g. nortriptyline and desipramine, the main differences being the absence of hydroxy metabolites and the presence of an acidic metabolite (Lu 4-028) of *Lu 3-010* Hydroxy-metabolites are formed in considerable amounts from imipramine and desipramine (CRAMMER & SCOTT 1966), and are assumed but not proven to be formed also from nortriptyline (MCMAHON *et al* 1963)

Lu 4-028 is found as the main metabolite A similar acidic metabolite has been found as degradation product of chlorpromazine, but in extremely small amounts (RODRIGUES & JOHNSON 1966)

The unchanged compound is excreted in large amounts after injection which agrees well with its rather low lipid water partition coefficient. However the amount of Lu 4-028 relative to unchanged compound increases with time after injection. Thus urine collected from dogs after long term administration mainly contains Lu 4-028 The metabolic pathway described seems to be the only important one in the two species studied.

There is no essential species differen[illegible] on of radio-activity following administration of [illegible] mice, [illegible] dogs. The isotope distribution agrees [illegible] by [illegible]HON *et al* (1963) on the [illegible] In HOREŠOVSKY *et al* [illegible] san[illegible] distribution patte[illegible] im[illegible] to and rabbits ([illegible]) injection (DINGE[illegible]

The primary amine Lu 4-004 was found in varying amounts in all tissue extracts. In the rat the concentration was found to be of the same order as the parent drug except in the brain, where the concentration of primary amine was greater than that of the parent drug.

From isotope data the biological half life of *Lu 3-010* and its metabolites was calculated to be 7 hours as compared to 2½ for imipramine and 9 for desmethylimipramine (DINGELL *et al* 1964)

The elimination of *Lu 3-010* in dogs and rats is rapid In both species about 50% of the administered dose is eliminated in the faeces and in the case of the dog this amount represents a non-biliary elimination The isotope concentrations found in the pancreas and the salivary glands are sufficiently high to suggest that elimination occurs via these glands. Elimination of the drug through the gastric mucosa is another possible route. In mice radioactivity was demonstrated in the pyloric part of the stomach 20 minutes after intravenous injection with no radioactivity in the fundus, as seen in fig. 9 This is in good agreement with results obtained with amitriptyline – ^{14}C, where radioactivity was found in the gastric mucosa within 5 minutes after injection (CASSANO *et al* 1965) It seems logical to assume that *Lu 3-010* is partly eliminated through the gastric mucosa in the dog.

Summary

The distribution, metabolism and excretion of a new bicyclic thymoleptic have been studied in rats, dogs and mice The distribution pattern shows large concentrations in the lungs, liver and kidneys and smaller concentrations in the brain fat and muscles, which is in good agreement with results published for tricyclic thymoleptics. The metabolic degradation proceeds by demethylation to primary amine followed by an oxidative deamination to a propionic acid derivative, which is the main metabolite found in the urine and faeces. The unchanged drug as well as metabolites are excreted in the urine and in the faeces in approximately equal amounts.

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From NOVO Terapeutisk Laboratorium,
Copenhagen Denmark

The Metabolic Fate of 5-(bicyclo-3, 2, 1-oct 2-en-2 yl)-5-ethyl Barbituric Acid, (Reposal ®).

By

P Nielsen and F Tarding

(Received M y 1968)

Studies on the metabolic fate of drugs in the human subject are of interest with regard to the evaluation of both the pharmacodynamic properties and the toxicity of a drug.

The present study was undertaken in order to investigate the metabolism of a new hypnotic substance 5-(bicyclo-3,2,1-oct 2-en-2 yl)-5-ethyl barbituric acid* (fig. 1), the pharmacological and clinical effects of which are described by FREY (1962) and by KESSING *et al* (1963a & b).

A method for the determination of reposal in serum and plasma has also been included in the study The method permits a combined estimation of reposal and its metabolite and the measurement of each of these substances separately

The present study shows that a breakdown product of reposal which can be isolated from urine following administration of reposal is identical with the product formed by the oxidation of reposal with chromic acid.

It further indicates that the metabolism of reposal involves oxidation in the bicyclic side chain, forming a keto group in the 4-position and leaving the double bond intact.

Methods and Results

Oxidation of reposal with chromic acid was carried out as follows

7 4 g of potassium dichromate dissolved in 100 ml of 1 N-H_2SO_4 was added to 20 g of reposal dissolved in glacial acetic acid. The solution was heated to approximately 65 for 24 hours.

* Trade name in Denmark Reposal ®.

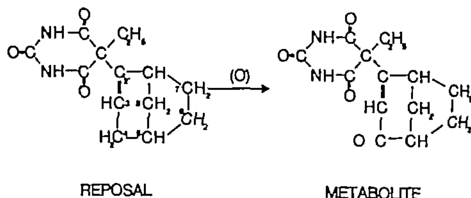


Fig. 1

Addition of distilled water to a final volume of 1 litre resulted in precipitation of white crystals. The crystals were isolated by filtration and washed with distilled water to remove any remnants of chromate.

Thin-layer chromatography (TLC) on silica gel plates (solvent diethyl ether) revealed that the crystalline precipitate consists of two components in approximately equal quantities, one of them being reposal, the other an oxidation product of reposal.

The two components were separated by column chromatography (diam. 3 cm, length 100 cm silica gel 0 05–0 20 mm Merck). The column was eluted with diethyl ether The substance, believed to be the product of oxidation appeared much less soluble than reposal e.g. in methanol (see table 2) Hence the oxidation product in subsequent preparations was isolated by repeated extractions of the mixture with methanol. After recrystallization from methanol the melting point was 281 (decomp) The result of elementary analysis of the oxidation product is shown in table 1 and corresponds to $C_{14}H_{16}N_2O_4$

Isolation of the metabolite from urine

Rabbits of the White Danish strain weighing approximately 2.5 kg and fasted for 18 hours were given reposal orally in doses of 100–200 mg/kg, 2–3 times at intervals of 4–5 hours.

The urine was collected throughout 24 hours after commencement of the treatment, in vials containing a few drops of toluene

The collected urine was acidified to pH 5 0 with hydrochloric acid and extracted continuously with ether The ether was removed by distillation. The brownish precipitate was recrystallized from methanol as white

Table 1

Elementary nalyses of reposal metabolite.

	Per cent			
	C	H	N	O
Oxidized reposal	61 00	5 92	10.15	2..97
Isolated f om urine	61 06	6.17	10.00	23 18
Theoretical for the metabolit ($C_{14}H$ N_2O_4)	60.87	5 80	10.15	23 18
2,4-Dinitrophenyl hydrazone.	52.40	4.53	18.35	24 72
Theoretical for the derivative ($C_{20}H_{20}N_4O_2$)	52.63	4.39	18 42	4.56

Table ?

Solubility of reposal and its met bolite at 25

Solvent	Reposal (g/100 ml)	Metabol te (g/100 ml)
Methanol	25	0.5
Ethanol	19	0 5
Acetone.	24	0 3
Diethyl ether	6	0.03
Chloroform	0.7	0 02
Distilled water (pH 6)	0.06	0 003
DMSO		10
Carbon tetrachloride		insol bl

crystals, m.p 281 (decomp) Elementary analysis gave the result shown in table 1 which corresponds to $C_{14}H_{16}N_2O_4$

Urine from patients given reposal was collected and extracted as described above. TLC of the ether extract in the media given in table 3 showed that the urine from humans contained a metabolite corresponding to that found in rabbit urine.

Following recrystallization from methanol the metabolite which can be isolated from the urine proved to be identical with the product of oxidation with chromic acid in as much as the melting point, the R_f values in TLC (Kieselgel GF_{254} nach Stahl Merck 0 25 mm), as well as the UV and IR spectra of the two substances (see below) proved to be identical.

Table 3

Rf-values for reposal and metabolite

Solvent		R posal	Metabolite	
			oxidation of reposal	isolation from urine
Benzene Ethanol abs. Diethylamine	8 pts 1 – 1 –	0.27	0.10	0.10
Benzene Ethylacetate Diethylamine	7 – 1 –	0.32	0.09	0.09
Benzene Dioxane Ethanol abs. Ammonia-conc.	10 – 8 – 1 – 1 –	0.48	0.05	0.05
Ethanol abs. Glac. acetic acid Distilled water	6 – 3 1	0.79	0.86	0.85
Chloroform Isopropanol Ammonia-conc.	9 9 2	0.5	0.[illegible]2	0.22
Chloroform Acetone	9 1	0.3	0.13	0.13
Diethyl ether		0.75	0.35	0.35

Quantitative estimation of the metabolite after treatment of urine samples with β-glucoronidase revealed that 20–40 % of an oral dose could be recovered in the urine as the above mentioned metabolite. The figure represents the results of three experiments in which volunteers were given 200 mg of reposal and their urine collected up to 18 hours after ingestion.

At this time no detectable amount of reposal or metabolite is present in the blood.

Physical and chemical properties of the metabolite

Table 2 illustrates the solubility of the metabolite which is readily soluble in dimethyl sulphoxide, soluble in methanol, ethanol, acetone, glacial acetic acid, slightly soluble in diethyl ether, chloroform and water (pH 6), and practically insoluble in carbon tetrachloride.

The metabolite dissolved in ethanol forms an orange coloured deriv-

ative with 2,4-dinitrophenyl hydrazine hydrochloride, which precipitates after cooling. Recrystallized from ethanol its melting point was 289 The elementary analysis is shown in table 1 and corresponds to $C_{20}H_{20}N_6O_2$

On spraying the TLC plates with a solution of 2,4-dinitrophenyl hydrazine, the metabolite reacts producing 2,4-dinitrophenyl hydrazone, while reposal does not react.

UV spectra were obtained with a Beckmann DB spectrophotometer As shown in fig. 2, the UV spectra of both reposal and the metabolite, dissolved in 0 45 N-sodium hydroxide solution, exhibit a peak around 255 mμ, typical for the barbituric acid ring. The difference in absorption of the two substances is at a maximum around 230 mμ.

IR spectra of reposal and the metabolite were recorded with a Perkin Elmer model 221 The IR spectra are shown in fig. 3 Unlike reposal the metabolite shows an additional peak around 1680 cm $^{-1}$ indicating the introduction of a keto group in the molecule.

The NMR spectroscopy* of reposal and the metabolite was performed with a Varian model A. 60 The compounds were dissolved in deuterated dimethyl sulphoxide.

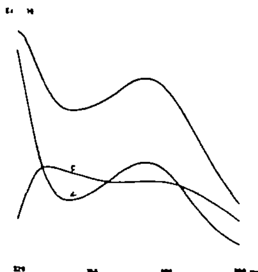


Fig. 2. Ultraviolet absorption spectra of reposal (A) and metabolite (B). Curve C represents the difference in absorption between A and B.

* We are indebted to Dr J Rastrup Andersen, 5th Department of Chemistry University of Copenhagen, for assistance in the performance of the NMR spectra and in interpretation of the results.

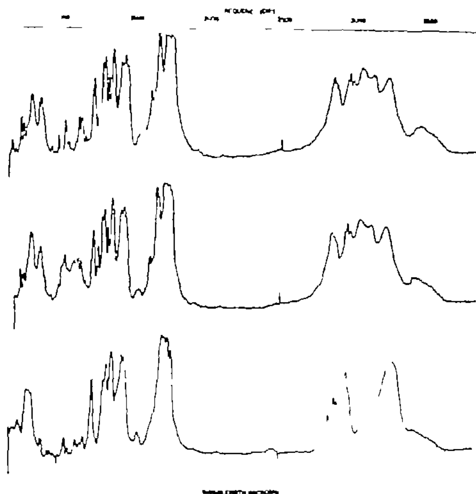


Fig. 3 Infrared absorption spectra of reposal (lower curve), metabolite isolated from urine (middle curve), and the chromic acid oxidation product of reposal (upper curve).

The NMR-spectra disclosed that the barbituric acid ring is intact in the metabolite. It is also apparent that the double bond in the side chain is intact in the metabolite, whereas changes have taken place at the carbon atom in the 4-position of the bicyclic side chain

TLC of reposal and the metabolite was performed using 0.25 mm silica gel plates (Kieselgel GF_{254} Stahl Merck) diethyl ether serving as solvent. The plates were developed by UV radiation (non-destructive) or by spraying with 0.1% potassium permanganate solution (destructive). These methods allow detection of between 1 and 10 µg of substance.

Table 3 shows the R_f-values obtained for reposal and the metabolite isolated from urine and from oxidation of reposal with chromic acid

These R_f data indicate that the best separation of the two substances is obtained with diethyl ether

Procedure for quantitative determination of reposal and metabolite

From the above mentioned findings a quantitative determination of reposal and the metabolite can be performed in the following way 2.0 ml plasma acidified by addition of 1 0 ml 4 N H_2SO_4 is extracted with 50.0 ml chloroform in a separating funnel.

The chloroform layer is separated and dried by filtration through dry sodium sulphate. The extraction is repeated twice with 5 0 ml of chloroform. For total analysis (reposal + metabolite) the combined chloroform extracts are extracted with 5 0 ml 0 45 N NaOH by vigorous shaking for 1 min. After separation of the phases the aqueous layer is cleared by centrifuging. The extinction at 255 and 275 mμ are read in a spectrophotometer The extinction difference between 255 and 275 mμ is proportional to the quantity of reposal and metabolite. In separate determinations of reposal and the metabolite the chloroform extraction is performed as above. The chloroform extract is evaporated to dryness on a water bath. The evaporation residue is dissolved in 250 μl methanol. 100 μl samples are placed on a TLC plate (Kieselgel GF_{254} nach Stahl, Merck, 0.25 mm, 20 × 20 cm) spaced 2 cm. Every second spot is a standard sample containing reposal and the metabolite. The plate is run with diethyl ether to a height of 10 cm. By illumination with a UV-lamp the spots for reposal and the metabolite are marked in the standard samples. The corresponding areas for the test sample are scraped off

The kieselgel is transferred to test tubes and extracted with 5 0 ml 0 45 N-NaOH by vigorous shaking for 1 min. After centrifugation the liquids are transferred to quartz cuvettes and the extinction at 255 and 275 mμ is measured as above.

The extinction difference is calculated and the concentration of reposal and metabolite determined by means of standard curves.

This procedure has been applied for the determination of the concentration of reposal, metabolite, and the total concentration of barbiturates in the plasma. The results are shown in fig. 4 The data represent the average of two experiments in dogs which were given 10 mg of reposal per kg intravenously

It is noteworthy that the concentration of the metabolite does not exceed 1–2 μg/ml and that this concentration is maintained throughout the experimental period

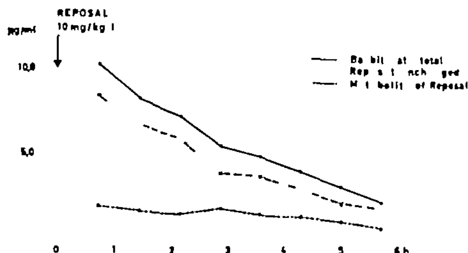


Fig. 4 Plasma concentration of reposal (unchanged), its metabolite and the total content of barbiturate after i.v administration to dogs (average of 2 exp.)

Biological activity of the metabolite

Reposal and the metabolite were administered orally to groups of mice weighing 20–25 g and the indicence of death was recorded The compounds were suspended in 1% Tween 80 The LD50 for reposal was 910 mg/kg (1001–827) The metabolite was administered in doses of up to 9000 mg/kg. No mortality was registered within 14 days after administration The metabolite appears to be much less toxic than reposal

The hypnotic effect of the metabolite was investigated after administration of up to 9000 mg/kg orally but no hypnosis was recorded. The 5 min. ED50 for reposal in corresponding experiments after oral administration is 110 mg/kg and after i.p injection 66 mg/kg (FREY 1962).

Discussion

From the above mentioned data it is suggested that the metabolic fate of reposal in the organism proceeds as indicated in fig. 1 from which it appears that an oxidation in the bicyclic side chain has taken place at position 4 The correctness of this suggestion is supported by the following facts

The NMR-spectra shows that the barbituric acid ring is intact in the metabolite as well as the double bond in the side chain. Comparing the spectrum of reposal with that of the metabolite it is apparent that changes have occurred at the carbon atom (position 4') next to the double bond atom (position 3').

The UV-spectra also indicate that the barbituric acid ring is intact. The absorption maximum at 255 mμ, characteristic of barbiturates, is retained. The difference in absorption between reposal and the metabolite is maximal around 230 mμ indicating that the metabolite contains an α,β-unsaturated keto group

From the IR-spectra it is also seen that the number of keto groups in the metabolite is increased compared with reposal. The elementary analysis supports this by giving $C_{14}H_{16}N_2O_4$, an increase in oxygen as compared with reposal ($C_{14}H_{18}N_2O_3$)

The metabolite is capable of forming a derivative with 2,4-dinitrophenyl hydrazine whereas this is not the case with reposal, further indicating that a new keto group is introduced in the molecule.

The metabolic alteration of reposal seems to follow one of the common schemes for barbiturate detoxication in the organism which involves oxidation of one of the radicals in the 5-position. For the compound in question an α,β-unsaturated ketone is formed in the bicyclic side chain

This metabolic fate of a barbiturate was first shown by FRETWURST *et al* (1932) who identified the keto-derivative of cyclobarbital (5-(cyclohexen-1-yl)-5-ethyl barbituric acid). The position of the keto group was later determined by TSUKAMOTO *et al.* (1955) The oxidation is analogous to other barbiturates with unsaturated cyclic radical in the 5-position, e.g. heptabarbital (5-(cyclohepten-1 yl)-5-ethyl barbituric acid) (PULVER 1943) and hexobarbital (5-(cyclohexen-1 yl)-1,5-dimethyl barbituric acid) (BUSH *et al.* 1953)

The keto-derivative is the major metabolic product of reposal, though other metabolites exist. This was shown by FREY (1962) who on paperchromatograms of human urine extracts, found three metabolites, of which one is presumably the keto-derivative

It is known from previous investigations (KESSING *et al* 1963a) that reposal has a rapid onset and a short duration of action This may be due to at least two factors The high degree of fat solubility (KESSING *et al.* 1963a) resulting in a rapid distribution within the body and the low *in vivo* stability of the drug. The bicyclic side chain is readily oxidized in the liver Consistent with this, only minimal quantities of reposal are excreted in the urine. The plasma concentration curves (Fig. 4) further indicate that the rate of elimination of reposal in the organism is governed by the oxidation capacity of the liver It is seen that the concentration of the metabolite is lower than that of reposal, only 1–2 μg/ml of the metabolite being detected in the blood independent of variation in the level of reposal.

It is reasonable to believe that this is due to differences in the chemical properties of reposal and the metabolite. Because of the high lipophility of reposal it is easily reabsorbed in the tubules of the kidney while the

more polar keto-derivative is much less reabsorbed. The solubility of the two compounds in question is also different, reposal having a 20-fold higher solubility in water than the metabolite.

The low toxicity and absence of hypnotic effect of the metabolite are properties also found in other primary barbiturate metabolites formed by side chain oxidation (TSUKAMOTO *et al* 1955 PULVER 1943 BUCH *et al.* 1953 RICHARDS & TAYLOR 1956).

Summary

A metabolite of 5-(bicyclo-3,2,1-oct 2-en-2 yl)-5-ethyl barbituric acid (Reposal ®), which can be isolated from urine in amounts of 20–40 / of an oral dose, is identified with the oxidation product of reposal with chromic acid (5-(bicyclo-3,2,1-oct 2-en-4-on-2 yl)-5-ethyl barbituric acid).

The chemical structure of the metabolite is discussed It is believed that oxidation in the bicyclic side chain takes place forming an α,β-unsaturated ketone

The metabolite has no hypnotic action and its toxicity is much less than that of reposal.

A procedure for analytical determination of reposal and the metabolite separately in biological media is described.

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5-Hydroxyindoleacetic Acid in Ventricular Cerebrospinal Fluid and Brain of Normal and Hydrocephalic Dogs after Administration of 5-Hydroxytryptophan. II

By

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(Received July 6, 1968)

In previous work (ANDERSSON & ROOS 1968) the level of 5-hydroxy indoleacetic acid (5-HIAA) in the cisternal cerebrospinal fluid (CSF) was determined at various intervals after the administration of the precursor 5-hydroxytryptophan (5-HTP). This investigation was made on normal dogs. It was shown that the maximal concentration of 5-HIAA in the cisternal CSF occurred about 3.5 hours after the injection of 5-HTP.

In dogs made hydrocephalic experimentally the level of 5-HIAA in the CSF was about twice the normal level (ANDERSSON 1968). This has been assumed to depend on either a decrease in the elimination of the acid metabolite from the CSF or on an increase in the synthesis and release of 5-hydroxytryptamine (5-HT) in the brain. The purpose of the present investigation was to get further information on these problems by determinating the 5-HIAA in the ventricular CSF after the administration of 5-HTP to dogs with experimental hydrocephalus.

Material and Methods

Twenty-one mongrel dogs of various ages were used for the experiments. In two untreated dogs the levels of 5-HT and 5-HIAA were determined in the caudate nucleus and in the posterior brain stem (i.e. medulla oblongata, pons and the posterior colliculi, fig. 1). The animals were kept under pentobarbital anesthesia during the experiments and killed by exsanguination before removal of the brain. In twelve dogs hydrocephalus was induced with technique described by ANDERSSON (1968) and the experiments were performed about two weeks later.

The degree of hydrocephalus was estimated from the quantity of CSF it was possible to remove from the ventricular system and the widening of the lateral ventricles and

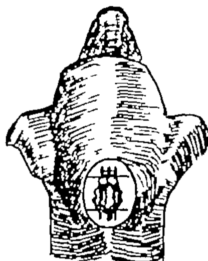


Fig. 1 Schematic drawing of posterior brain stem" The cerebellum is removed.

Sylvian aqueduct. For practical purposes methods for ventricular measurement involving formalin treatment of the brain (GRANHOLM 1966) could not be used.

After the intravenous injection of 5-HTP (25 mg/kg bodyweight) into four normal and six hydrocephalic animals, 1.0 ml of ventricular CSF was withdrawn at regular intervals by puncture of a lateral ventricle. With the method used for the induction of hydrocephalus in this investigation it is extremely difficult to get CSF from the cisterna magna. The cisterna magna is nearly always totally blocked by the changes caused by kaolin. On two occasions, however it was possible to obtain cisternal CSF from hydrocephalic dogs. The level of 5-HIAA at the end of the experiments was determined in the caudate nucleus and in the brain stem.

The concentration of 5-HIAA in the CSF and brain tissue was determined according to the methods of ASHCROFT & SHARMAN (1960), ROOS (1963) and WERDINIUS (1967). For more detailed information on the experimental procedure see ANDERSSON (1968) and ANDERSSON & ROOS (1968) The 5-TH was determined ccording to ANDÉN & MAGNUSSON (1967).

Results

The ventricular CSF value of 5-HIAA in two normal dogs was 0.13 and 0 17 μg/ml. The mean CSF value of 5-HIAA in the normal ventricles (five dogs) in a previous investigation was 0.21 ± 0.03 μg/ml (S.E.M) (ANDERSSON 1968). These values might be compared with the corresponding values in the present investigation in two normal dogs i e. of 0.17 μg/ml and 0 19 μg/ml, respectively When these values are included the mean value in our calculation is 0.19 ± 0 02 μg/ml (S E.M) (seven dogs).

After the intravenous injection of 5-HTP in to four normal dogs there was a moderate increase in the level of 5-HIAA in the ventricular CSF

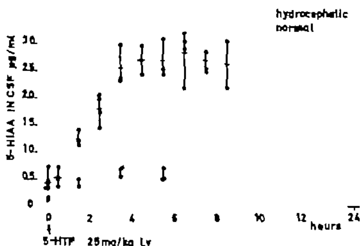


Fig. 2. The concentration of 5-HIAA in ventricular CSF at various intervals after i.v. injection of 5-HTP (25 mg/kg). Open circles – hydrocephalic dogs. Closed circles – normal dogs.

(fig. 2) In two of them a rise in the concentration was noted about seven and a half hour after the injection of the precursor. Complete return of the metabolite to normal levels was not observed within 12 hours.

The mean ventricular CSF level of 5-HIAA in six hydrocephalic dogs before the administration of 5-HTP was 0.46 ± 0.06 μg/ml (S.E.M.).

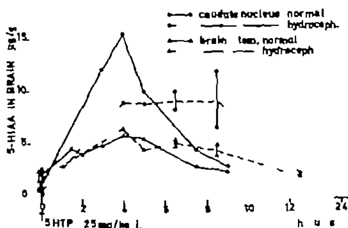


Fig. 3. The concentration of 5-HIAA in the brain tissue at various intervals after i.v. injection of 5-HTP (25 mg/kg). Open symbols – hydrocephalic dogs. normal dogs.

Table 1

Mean concentration of 5-HT and 5 HIAA in normal and hycrocephalic dogs (brain stem (B.S.) and caudate nucleus (C.N)) before and after intravenous injectio of 5-HTP

	Normal value		2 hrs. after L 5-HTP	
	C.N	B.S	C.N	B.S.
5-HT				
Controls	0.24 ± 0.04 (2)	0.50 ± 0 16 (2)	4.38	1.85
Hydrocephalic	0.20	0.39	4.05	0.90
5-HIAA				
Controls	0.62 ± 0.05 (2)	1.90 0.44 (2)	7 73	4 15
Hydrocephalic	0.53 ± 0.15 (4)	1.62 ± 0.53 (4)	7 96	3.94

After the intravenous injection of 5-HTP into the hydrocephalic dogs there was a pronounced increase of 5-HIAA in the ventricular CSF The maximal concentration was reached 3 to 4 hours after the injection and appeared to remain at this level for the following five hours, after which there was a decrease of the acid to the initial level within twenty-four hours (fig. 2) In the two cases in which it was possible to obtain CSF both from the lateral ventricle and from the cisterna magna the values of 5-HIAA in the ventricular fluid were 0.57 and 0 41 μg/ml, and in the cisternal fluid 0.36 and 0.42 μg/ml, which gives ventriculo-cisternal ratios of 1 6 and 1 0, respectively

The increase in 5-HIAA after 5-HTP in the normal brain was relatively rapid with a maximum between 3–5 hours after the injection of 5-HTP while the corresponding increase in the hydrocephalic brain was slightly slower the maximal concentration being lower and also the decrease being slower and giving a prolonged curve for about 8 hours (fig. 3).

The values of 5-HT and 5-HIAA in the brains of the normal and the hydrocephalic dogs before and after intravenous injection of 5-HTP are shown in table 1 and fig. 3

Discussion

When comparing normal and hydrocephalic dogs, there was a significant difference in the values of 5-HIAA in the ventricular CSF before the administration of 5-HTP

Three and a half hour after the injection of 5-HTP to normal dogs the values of 5-HIAA in the ventricle and the cisterna (ANDERSSON & ROOS

1968) were about 0 6 μg/ml showing a nearly equal increase in the cisternal and ventricular concentrations of the metabolite the same time however the increase in 5-HIAA in the hydrocephalic ventricle was nearly four times higher (2.5 μg/ml) (fig. 2) which is highly significant.

The experiments have thus shown two important facts. The increase in 5-HIAA after the intravenous injection of 5-HTP was more rapid and much more pronounced in the ventricular CSF of hydrocephalic dogs than in normal dogs (fig. 2) On the contrary the increase and decrease of 5-HIAA in the brain was slower in the hydrocephalic dog and further more the concentration did not reach the levels found in normal dogs (fig. 3).

There are three main possible explanations for the enhanced 5-HIAA accumulation in the CSF of hydrocephalic dogs treated with 5-HTP

1 an increased formation of 5-HIAA.
2. a facilitated efflux of 5-HIAA from the brain tissue to the CSF
3. a decreased elimination of 5-HIAA from the CSF

Against the first assumption of an increased formation of 5-HIAA in hydrocephalus is the fact that the initial concentrations of 5-HT and 5-HIAA are apparently the same in normal and hydrocephalic dogs. Moreover after the injection of 5-HTP the values of 5-HT and 5-HIAA are lower in hydrocephalic than in normal dogs It might therefore be reasonable to assume that there are no obvious changes in the synthesis and turnover of 5-HT in the hydrocephalic brain.

The second hypothesis of a facilitated efflux of 5-HIAA from brain tissue to CSF is more difficult to reject. This intravenously administered 5-HTP appears to penetrate to some extent directly into the CSF (ANDERSSON & ROOS 1968) The enzymes necessary for the formation of 5-HT and 5-HIAA are only found in brain tissue and we have therefore assumed that the 5-HTP from the CSF enters the brain quickly Normally 5-HIAA does not readily cross the blood brain barrier (ROOS 1962), neither does this seem to occur with 5-HT except in very high doses (BULAT & SUPEK 1967) In hydrocephalus on the other hand not only is the passage of 5-HIAA across the blood-brain barrier but also from the blood to the CSF possible However no direct passage from blood to ventricular CSF was observed in hydrocephalic dogs 0.5–5 hours after intravenous injection of 5-HIAA, 1 mg/kg (ANDERSSON & ROOS, unpubl.). It thus seems likely that the concentration of 5-HIAA in the CSF mainly depends on 5-HIAA transfer from brain tissue to CSF and secondarily on the elimination of 5-HIAA from the CSF Probably only a fraction of 5-HIAA in the brain is removed by passage to CSF The major part seems disappeared in the blood (NEFF *et al* 1967). If the increase in CSF

in hydrocephalus after intravenous loading with 5-HTP does depend on facilitated efflux of 5-HIAA from the brain to the CSF it is difficult to explain why the level of 5-HIAA in the brain decreases more slowly than in the normal brain.

The initial concentration of 5-HIAA and 5-HT was found to be higher in the brain stem than in the caudate nucleus in agreement with the findings of e.g. ECCLESTON *et al* (1968) After the intravenous injection of 5-HTP there was a reversed distribution, with about twice the concentration in the caudate nucleus as in the brain stem, four hours after injection of the precursor This reverse effect was also found in the hydrocephalic brain even though not as marked. This might depend on a high concentration of 5-HTP decarboxylase in the caudate nucleus as compared with the brain stem (UDENFRIEND 1956) It also shows that the nerve terminals in the caudate nucleus of the hydrocephalic dog behave *almost normally with regard to the decarboxylation of 5-HTP*

The remaining alternative for the findings in hydrocephalic CSF is a decreased elimination of 5-HIAA from the CSF in hydrocephalus. If the elimination is diminished it could be expected that loading with the precursor would increase the concentration of 5-HIAA in the CSF more than normally The investigation thus supports the view even though it does not prove it, that there is a disturbance in the elimination of 5-HIAA. No observations were made to contradict this hypothesis.

Further evidence in favour of a decreased elimination in hydrocephalus was obtained by comparing the concentrations of 5-HIAA in the ventricular CSF and the cisternal CSF It has previously been reported (ANDERSSON & ROOS 1968) that the mean value of 5-HIAA in cisternal CSF in normal dogs is 0 07 $\pm$ 0 005 μg/ml (S E.M) (24). The mean value of 5-HIAA in the ventricular CSF in the present investigation was 0.19 $\pm$ 0 02. The ventriculo-cisternal ratio of 5-HIAA in the CSF of normal dogs can thus be calculated to 2.7 which is lower than the ventriculo-cisternal ratio reported by GULDBERG *et al* (1966) (probably due to different methods in obtaining CSF) Differences in the dog strain might be relevant. However the important finding is a higher value of 5-HIAA in the ventricular CSF as compared with the cisternal CSF in the normal dog. The reason for this difference is not clear but probably 5-HIAA in the CSF is mostly derived from brain substance, the larger brain ependymal surface being exposed to the ventricles than to the cisterna. It has also been suggested that the site of elimination of acid compounds in general as well as this particular acid metabolite, is mainly located in the fourth ventricle and in the cisterna magna (PAPPENHEIMER *et al.* 1961 GULDBERG *et al.* 1966) On two occasions it was possible to obtain cisternal CSF from hydrocephalic dogs. These dogs had ventriculo-cisternal ratios of

1 6 and 1 0 respectively The ratio in these hydrocephalic dogs was thus lower than that in normal dogs.

In this type of hydrocephalus in which the cisterna and ventricles form a closed unit it could be expected that the ratio would tend to approximate 1 0 providing the 5-HIAA enters the CSF mainly via the ventricle and the elimination from the cisterna is diminished After the administration of 5-HTP to the normal dog there was a similar increase of 5-HIAA in the ventricle and cisterna (ANDERSSON & ROOS 1968) up to 0.6 μg/ml three and a half hour after the injection of the precursor This might be explained by overloading of the mechanism for elimination. It would also have been of interest to see if there was the same tencency for a parallel increase in the hydrocephalic animals after 5-HTP but unfortunately it was not possible to obtain cisternal CSF from the hydrocephalic dogs in this series.

Further evidence in favour of a decreased elimination was obtained in an investigation on the elimination of intraventricularly injected 5-HIAA, when it was found that the decrease in the concentration of 5-HIAA was slower in the hydrocephalic dog (ANDERSSON & ROOS to be published)

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Summary

5-Hydroxytryptophan (5-HTP) the precursor of 5-Hydroxytryptamine (5-HT) was given intravenously to normal dogs and dogs with experimentally induced hydrocephalus. The concentration of 5-Hydroxyindoleacetic acid (5-HIAA) in the cerebrospinal fluid (CSF) the caudate nucleus and the brain stem was determined at various intervals after the injection of 5-HTP

A rapid and pronounced increase in 5-HIAA was found in the CSF of hydrocephalic dogs but the increase of the metabolite in the brain substance was slower and more prolonged than normal in the hydrocephalic animal These findings are discussed and support the view of a decreased elimination of 5-HIAA from the CSF in experimental hydrocephalus. Comparisons between the ventriculo-cisternal levels of 5-HIAA also favour this hypothesis.

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Passage of ^{14}C Nicotine and Its Metabolites into Mice Foetuses and Placentae

By

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(Received May 20, 1968)

Various effects of nicotine and/or smoking on the foetus of animals and man have been reported by several investigators (for review see LARSON *et al.* 1967) Among the effects observed are a retardation of foetal growth of infants whose mothers smoked (LOWE 1959), and signs of immature development of the foetus after nicotine administration to pregnant rats (BECKER & KING 1966) LOWE suggested that smoking caused a restriction of placental circulation thus limiting the blood supply to the foetus

That nicotine passes from the mother to the foetus has been shown by several investigators (e.g. NAKASAWA 1931 & 1933 MORRA 1935 ROMANIELLO 1939 SERGUEEV 1939 WERLE & MEYER 1950 DOERFEL 1952) More recently HANSSON & SCHMITERLÖW (1962) using ULLBERG's whole body autoradiographical technique, observed the passage of ^{14}C nicotine and/or its metabolites into the foetuses of pregnant mice

The present paper presents investigations on the amount of nicotine and/or its metabolites that passes into the foetus when ^{14}C-nicotine is administered to pregnant mice It also deals with the rate of passage, the amount of metabolites in the foetus and with the question whether foetal tissues are capable of metabolizing nicotine

Material and Methods

Experimental animals

White pregnant mice of the NMRI-strain weighing between 34 and 45 g were used in the investigations. Unless otherwise stated the mice were 16–18 days pregnant.

^{14}C-labelled nicotine

Nicotine methyl-^{14}C was synthetized as previously described (M. Kennedy, Hansson & Schmiterlöw 1962). The specific activity was 57.33 µcl/mg.

Whole-body autoradiography

[14]C-nicotine was injected intravenously and subcutaneously in dose of 0.14 μCi/g body weight, corresponding to 2.44 μg nicotine/g body weight.

The mice were sacrificed at 5 min., 15 min., 30 min., 1 hour and 4 hours after injection by anaesthetizing with ether and immersion in hexane cooled with solid carbon dioxide to about −70°.

Sagittal sections (30 μ and 80 μ thick) were cut through the whole frozen animals in a refrigerated room at about −10°. Autoradiography was performed as described by ULLBERG (1954). The sections adhering to an adhesive Scotch tape were dried in the same refrigerated room and pressed on Kodirex (Kodak) X-ray film (80 μ sections) and on Structurix (Gevaert) X-ray film (30 μ sections). The exposure time varied between 15 and 90 days.

Procedure for isolation of nicotine and metabolites in tissues

[14]C-nicotine was injected intravenously and subcutaneously. The injected dose was 0.041 μCi/g body weight, corresponding to 0.71 μg nicotine/g body weight.

The mice were sacrificed 5 min., 15 min., 30 min., 1 hour and 4 hours after the injection by decapitation after anaesthetizing with ether.

Maternal blood, placentae and foetuses were used for metabolic investigations. The brains and livers were also removed from the intravenously injected animals, for comparison with blood, placental and foetal concentrations.

Nicotine and cotinine were extracted, with modifications, according to the method originally described by HUCKER *et al.* (1960).

The radioactive nicotine and cotinine in the heptane and chloroform phase were determined in a Packard Tri-Carb liquid scintillation counter after the addition of 9 ml ethanol + 9 ml toluene with 10 g PPO per 1 toluene.

After the nicotine and the cotinine had been extracted from the homogenate, 4 ml of distilled water was added. The flask was shaken for 10 minutes and centrifuged, and 0.1 ml of the water phase was removed and used for the radioactive determination of water soluble nicotine metabolites in the Packard Tri-Carb liquid scintillation counter following the addition of 9 ml ethanol and 9 ml toluol with 10 g PPO/l toluol.

The extraction procedure for nicotine and cotinine was confirmed by the use of thin-layer chromatography.

A separate experiment was performed to identify the radioactivity in various foetal organs. An 18-day-pregnant mouse was injected with [14]C-nicotine subcutaneously (0.041 μCi/g corresponding to 0.71 μg nicotine) and sacrificed 30 minutes after the injection. A whole foetus, and the livers, the placentae and the lungs of some (6–8) foetuses were homogenized in 2 ml Krebs-Henseleit buffer and the homogenates extracted with chloroform-methanol (2:1). The chloroform-methanol phase was removed and evaporated to dryness. The residue was again dissolved in a small amount of chloroform-methanol which was added to thin-layer plates for chromatography.

Thin-layer chromatography

Thin-layer chromatography was performed on silica gel plates together with reference compounds, using ethanol-acetone-benzene-conc. NH_4OH (5:40:50:5) as solvent. Silica gel thin-layer plates were prepared as described by STAHL (1962) and were activated by heating at 110° for 30 minutes. Radioactive compounds were located by exposing the plate to Kodak No-screen X-ray film.

Incubation of tissue slices with ^{14}C-nicotine

Tissue slices of the mother's liver placentae, as well as th livers and lungs of 18-day-old foetuses ere prepared by hand with a razor blade and incubated in Krebs-Henseleit phosphate buffer pH 7.4 containing glucose t concentration of 0.01 M The experiments ere performed in Dubnoff shaker at 37° The slices were incubated with ^{14}C nicotine (0.1 µci) for two hours in an atmosphere of oxygen.

After incubation the tissues were homogenized and the homogenate extracted with chloroform-methanol (2:1). The component of the chloroform-methanol extract were separated by thin-layer chromatography

Placental transfer at various periods of pregnancy

Two groups of NMRI-mice, 14 16 and 18 days pregnant, were injected with 0.020 µci/g ^{14}C-nicotine corresponding to 0.355 µg/g ^{14}C-nicotine, intravenously and subcutaneously The animals were sacrificed 30 minutes after th injection and the blood fr m the mother the placentae and the foetuses were rem ved. The radioactive components i the tissues were separated and estimated as described above.

Results

General distribution

Autoradiographic investigation The general tissue distribution has been described in detail in a previous paper from this department (HANSSON & SCHMITERLÖW 1962) and only a short description will be given here

The autoradiograms (fig. 1–4) reveal that immediately after the injection there is a high concentration of radioactivity in the brain This concentra tion then rapidly diminishes and by 30 minutes there is little radioactivity left in the brain.

A rapid concentration of radioactivity can also be seen in the liver the lungs, the kidneys, the salivary glands, the fundus of the stomach the adrenal glands and the pituitary gland. In the mammary gland a rather high concentration can be seen between 15 minutes and 1 hour after the injection. The concentration in the foetal tissues is low during the whole observation period.

Quantitative determinations of the concentration of total radioactivity in the liver brain, blood and foetuses of intravenously injected mice are seen in fig. 5

In the blood brain and liver the highest concentration of total radioactivity is seen within the first 5 minutes. The concentration decreases rapidly during the first 30 minutes after the injection and thereafter only a slight decrease occurs.

In the foetus the highest concentration of radioactivity is seen 30 minutes after the injection. The radioactivity then decreases gradually

Fig. 6 shows that the rapid accumulation of radioactivity observed in the brain is to a large extent due to nicotine while the radioactivity in the

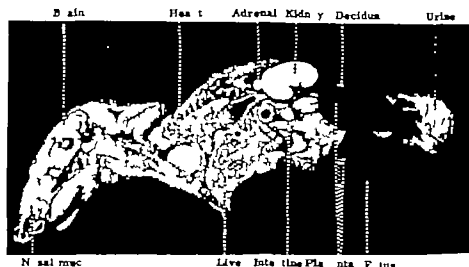


Fig. 1 Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 5 minutes after i.v. injection of ^{14}C-nicotine. Sagittal section through the whole body of the animal. High radioactivity can be seen in the brain, the nasal mucosa, the kidney and the adrenal of the mother. No radioactivity can be seen in the foetus. The placenta contains a small concentration and the decidua basalis high concentration of radioactivity

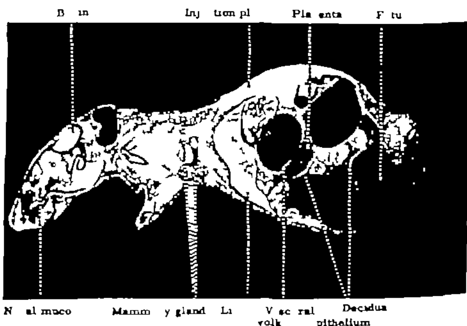


Fig. 2. Autoradiogram showing the distribution of radioactivity (light areas) in mouse 15 minutes after s.c. injection of ^{14}C-nicotine. Sagittal section through the whole body of the animal. High radioactivity can still be seen in the brain and at the site of injection. Radioactivity can be seen the foetus. Radioactivity is seen in the decidua basalis and in the part of the visceral yolk sac epithelium adjacent to the chorioallantoic placenta.

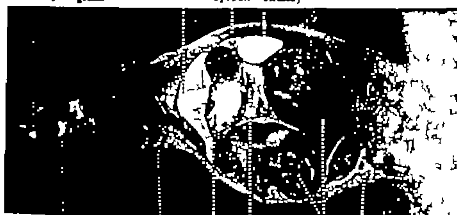


Fig 3. Autoradiogram showing the distribution of radioactivity (light areas) in mouse 30 minutes after s c. injection of ^{14}C-nicotine. Sagittal section through the whole body of the animal. The liver the gastric mucosa, the kidney Harder's gland and the salivary glands of the mother contain a good deal of radioactivity The concentration of radioactivity in the foetus is high compared with the foetus with a short survival time. For detail of a foetus see fig. 7

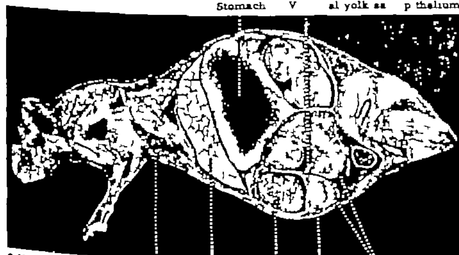


Fig. 4. Autoradiogram showing the distribution of radioactivity (light areas) in mouse 1 hour after s.c. injection of ^{14}C-nicotine. Sagittal section through the whole body f the animal. High radioactivity is seen in the liver and the gastric mucosa. Moderate radioactivity is seen in the foetus. The decidua basalis and parts of the visceral yolk sac epithelium have a high concentration of radioactivity The chorioallantoic placenta contains a moderate amount of radioactivity

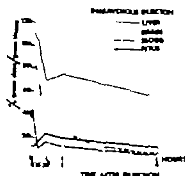


Fig. 5 Amount of radioactivity in the liver brain, blood d foetus after i. injection of ^{14}C-nicotine.

liver represents mainly metabolites. This can also be seen from table 1 where the relation between nicotine, cotinine and water soluble metabolites in the mother s liver and brain is given The relation between the placenta foetus and blood is given in the same table (see below)

Foetal and placental distribution

Autoradiographic investigation. Five minutes after the injection (intravenously or subcutaneously) no radioactivity can be seen in the foetus. In the placenta the amount of radioactivity is small. In the decidua basalis a rather high concentration can be seen (fig. 1) Fiften minutes after the injection (intravenously or subcutaneously) the concentration in the placenta and in the foetus has increased considerably In the decidua basalis the high concentration persists and in the part of the yolk sac, adjacent to the chorioallantoic placenta, a high concentration is also noted (fig. 2).

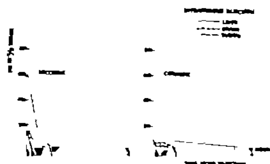


Fig. 6. Nicotine nd cotinine concentrations in the liver brain nd blood of mice at various times after i. injection of ^{14}C-nicotine.

Table 1

The radioactivity in per cent, of nicotine, cotinine and metabolites in the water phase of the placenta, foetus, blood, liver and brai of the mother at different survival times after i.v. injection of nicotine into pregnant mice. Th corresponding concentrations of the nicotine and the cotinine are given in fig. 6 and fig. 10.

	Time after injection	nicotine	% cotinine	% in the water phase
Placenta	5 min.	74.1	18.7	7.2
	15 min.	58.	27.9	13.9
	30 min.	44.5	32.6	22.9
	1 hr	24.1	19.1	56.8
	4 hr	3	13.6	83.2
Foetus	5 min.	59.4	34.4	6.2
	15 min.	42.0	40.7	17.3
	30 min.	32.0	42.8	25.2
	1 hr.	15.1	29.2	55.7
	4 hr.	2.6	20.0	77.4
Blood of mother	5 min.	46.5	35.7	17.8
	15 min.	16.6	41.6	41.8
	30 min.	10.0	33.4	56.6
	1 hr.	3.8	21.8	74.4
	4 hr	0	13.9	86.1
Liver of mother	5 min.	24.9	33.2	41.9
	15 min.	14.4	37.2	48.4
	30 min.	6.2	36.9	56.9
	1 hr	1.5	14.6	83.9
	4 hr	2.8	6.6	90.6
Brain of mother	5 min.	88.0	7.9	4.1
	15 min.	81.8	13.6	4.6
	30 min.	57.2	28.6	14.2
	1 hr	32.3	17.5	50.[illegible]
	4 h	5.0	13.6	81.4

Thirty minutes after the injection (intravenously or subcutaneously) the foetal and placental concentrations are still high. In the foetus a high concentration can be seen in the lungs and also in the trachea and in the larynx. A high concentration can also be seen in the foetal adrenal, kidney and intestine (fig. 3 7 8).

One hour after the injection (intravenously or subcutaneously) radioactivity still persists in the placenta and in the foetus. The decidua basalis

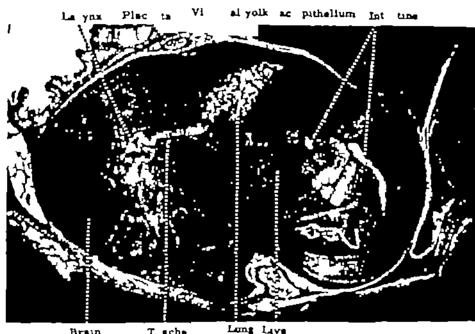


Fig. 7 Enlargement of a foetus (from fig. 3). High radioactivity is seen in the lung, the trachea, the larynx and the intestine. No accumulation of radioactivity can be seen in the brain and the liver

shows a high concentration and a high concentration is also seen in the part of the yolk sac adjacent to the chorioallantoic placenta (fig. 4).

Four hours after the injections the concentrations in the placenta and in the foetus are very low

Quantitative determination. The results of the metabolic investigations are given in fig. 9–12 and in table 1 and 2. The total radioactivity (fig. 9) in the placenta after intravenous injection reaches the highest level between 15 minutes and one hour after the injection. In the foetus concentration is lower than in the placenta with the highest amount occurring after 30 minutes In the blood there is also a peak after 30 minutes although the highest concentration here is reached immediately after the injection. The peak after 30 minutes is mainly due to nicotine metabolites (fig. 10, table 1) After intravenous injection (fig. 10) the highest concentration of nicotine in the placenta is reached after 15 minutes, while the highest concentration of cotinine is reached after 30 minutes

In the foetus the nicotine concentration is low during the whole period the highest concentration is reached after 30 minutes. As in the placenta the highest concentration of cotinine in the foetus is reached 30 minutes after the injection



Fig. 8. Autoradiogram showing the distribution f radioactivity (light areas) in two mouse foetuses 30 minutes after s.c. injection of 14C-nicotine. Detail of sagittal section through the whole body of an animal. In the foetuses high radioactivity is seen in the lung, the kidney the adrenal and the intestine. The part of the yolk sac epithelium djacent to the chorioallantoic placenta contains mor radioactivity than the chorioallantoic placenta itself

In the blood the nicotine concentration decreases rapidly and after 15 minutes there is only a very small quantity of nicotine in the blood. The cotinine concentration in the blood also decreases rapidly but

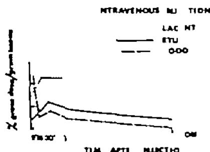


Fig. 9 Amount of radioactivity in the placenta, foetus and blood after i.v. injection of 14C-nicotine.

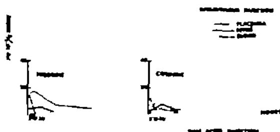


Fig. 10. Nicotine and cotinine concentrations in the placenta, foetus and blood f mice at various times after i. injection of ^{14}C-nicotine.

remains slightly higher than the nicotine concentration and there is a greater amount of cotinine after 30 minutes than after 15 minutes. Table 1 shows the relative decrease of nicotine and increase of water soluble metabolites in the placenta, blood and foetus. One hour after the injection the metabolites in the water phase dominate in all three cases. The relatively highest cotinine concentration is seen in the placenta and foetus after 30 minutes, and in the blood after 15 minutes It can also be seen, as mentioned above, that in the liver cotinine and water soluble metabolites become predominant early after the injection while in the brain, nicotine dominates for a longer time

The total radioactivity in the placenta after subcutaneous injection reaches a very high concentration after half an hour (fig. 11). In the

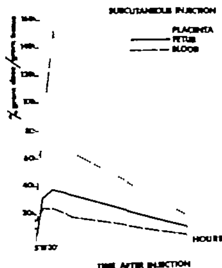


Fig. 11 Amount of radioactivity in the placenta, foetus and blood after s.c. injection of ^{14}C-nicotine.

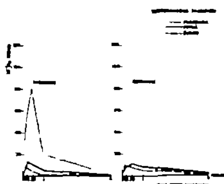


Fig. 12. Nicotine and cotinine concentrations in the placenta, foetus and blood of mice at various times after s.c. injection of ^{14}C-nicotine.

foetus the highest concentration is also reached after 30 minutes but this concentration is considerably lower than in the placenta. The blood concentration is again lower than the concentration in the foetus the highest level being reached after 15 to 30 minutes.

Table 2

The radioactivity in per cent, of nicotine, cotinine and metabolites in the water phase in the placenta, foetus and blood of the mother at different survival times after s.c. injection of nicotine into pregnant mice. The corresponding concentrations of nicotine and cotinine are given in fig. 12.

	Time after injection	% nicotine	% cotinine	% in the water phase
Placenta	5 min.	81 6	4.8	13 6
	15 min.	79 7	14.3	5 0
	30 min.	73.1	14.6	1[illegible]3
	1 hr	45.9	17 0	37 1
	4 hr	3.0	14.8	82.2
Foetus	5 min	67.0	11.9	21 1
	15 min.	56.3	33.0	10.7
	30 min.	42.3	40.8	16.9
	1 hr	24 1	32.9	43.0
	4 hr	3 6	23 6	72.8
Blood of mother	5 min.	84.3	15.7	0
	15 min.	44 4	55 6	0
	30 min.	2[illegible]3	47.8	29.9
	1 hr	11 7	39 7	48.6
	4 hr	0	15.9	94 3

Table 3

Total radioactivity in % gram dose/gram tissue in the placenta, foetus and blood of 14–18 days pregnant mice, killed 30 minutes after i.v. and s.c. injection of nicotine.

Day of pregnancy		% gram dose/gram tissue Radioactivity in		
		placenta	foetus	blood
Intravenous injection	14	42.2	25.1	7.6
	16	61.8	28.1	3.7
	18	49.3	15.6	4.6
Subcutaneous injection	14	73.3	36.3	7.5
	16	61.0	30.2	8.3
	18	63.8	35.2	7.8

After subcutaneous injection (fig. 12) there is a very high concentration of nicotine in the placenta between 15 minutes and one hour with a high peak after 30 minutes

The nicotine concentration in the foetus is much lower with the highest value after 15 minutes. In the blood there is a constant decrease in nicotine concentration and after one hour the amount of nicotine is low

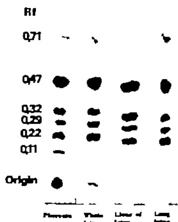


Fig. 13 Autoradiogram of thin-layer chromatogram of the chloroform phase of the the placenta, whole foetus, liver of foetus and lung of foetus. The pregnant mouse was injected s.c. with ^{14}C-nicotine and killed 30 minutes after injection. Solvent system ethanol-acetone-benzene-conc. NH_4OH (5 40 50 5). Components Nicotine (R_f 0.71), cotinine (R_f 0.47) γ-(3-pyridyl)-γ-oxo-N-methylbutyramide (R_f 0.32), "X" (R_f 0.29), hydroxycotinine (R_f 0.22), "Y" (R_f 0 11) (According to HANSSON *et al.* 1964).

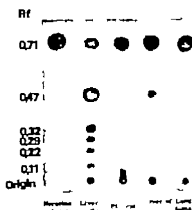


Fig. 14. Autoradiogram of thin-layer chromatogram of chloroform phase of slices from liver of the mother placenta, liver of foetus and lung of foetus. The slices were incubated with ^{14}C-nicotine. Solvent system: ethanol-acetone-benzene-conc. NH_4OH (5 40 50 5). Components See fig. 13

The cotinine concentration in the foetus and the placenta after subcutaneous injection shows a similar picture as after intravenous injection the highest amount of cotinine being present after 30 minutes (fig. 12). In the blood the highest concentration of cotinine is reached after 15 minutes.

From table 2 it can be seen that the relative concentration of nicotine is higher for a longer period after the injection than was the case in the mice injected intravenously The metabolites in the water phase do not become predominant until one to four hours after the injection.

Penetration at various periods of pregnancy

The amount of nicotine and nicotine metabolites in the mice foetuses at 14 to 18 days of pregnancy is seen in table 3 There does not seem to be any relation between the concentration of radioactivity and period of pregnancy thus indicating that the penetration is probably the same during the last 4 days of pregnancy

Nicotine metabolism in placenta and foetus

The chromatography plates show that the metabolites found in the placenta and the foetus after *in vivo* metabolism of nicotine can also be found in the liver of the mother after *in vitro* incubation with nicotine (fig. 13 and 14)

After *in vitro* incubation of the liver of the foetus it can be seen that the liver metabolizes nicotine to cotinine, but only to a small degree

Table 3

Total radioactivity in % gram dose/gram tissue in the placenta, foetus and blood of 14–18 days pregnant mice, killed 30 minutes after i.v. and s.c. injection of nicotine.

Day of pregnancy		% gram dose/gram tissue Radioactivity in placenta	foetus	blood
Intravenous injection	14	42.2	25.1	7.6
	16	61.8	28.1	3.7
	18	49.3	15.6	4.6
Subcutaneous injection	14	73.3	36.3	7.5
	16	61.0	30.2	8.3
	18	63.8	35.2	7.8

After subcutaneous injection (fig. 12) there is a very high concentration of nicotine in the placenta between 15 minutes and one hour with a high peak after 30 minutes.

The nicotine concentration in the foetus is much lower with the highest value after 15 minutes. In the blood there is a constant decrease in nicotine concentration and after one hour the amount of nicotine is low.

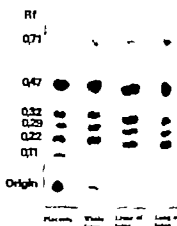


Fig. 13. Autoradiogram of thin-layer chromatogram of the chloroform phase of the the placenta, whole foetus, liver of foetus and lung of foetus. The pregnant mouse was injected s.c. with ^{14}C-nicotine and killed 30 minutes after injection. Solvent system: ethanol-acetone-benzene-conc. NH_4OH (5:40:50:5). Components: Nicotine (R_f 0.71), cotinine (R_f 0.47), γ-(3-pyridyl)-γ-oxo-N-methylbutyramide (R_f 0.32), "X" (R_f 0.29), hydroxycotinine (R_f 0.22), "Y" (R_f 0.11). (According to HANSSON *et al.* 1964).

The concentration of nicotine in the placenta is high compared with that in the foetus. The placenta continues to accumulate nicotine from the blood even after the blood concentration has become considerably lower than that of the placenta. The explanation of this may reflect transport from the decidua basalis into the placenta.

Although the structure of the placenta changes during pregnancy the penetration of nicotine seems to be unaffected from the 14th to the 18th day of pregnancy

The large accumulation of radioactivity in the visceral yolk sac epithelium nearest the chorioallantoic placenta has been observed with other compounds (BERLIN & ULLBERG 1963 APPELGREN *et al.* 1966 ULLBERG *et al.* 1967). The mechanism responsible for this accumulation is not clear (BÄCKSTRÖM *et al.* 1967)

In the foetus there is a higher concentration of radioactivity in the lungs trachea, larynx and in the adrenals, kidneys and intestine.

Little radioactivity is found in the liver An accumulation in the lungs, the kidneys, the adrenals and the intestine is also seen in the mother

A possible explanation for the fact that no accumulation of radioactivity is seen in the liver of the foetus may be that the metabolizing function of the liver of the foetus is very limited

From the chromatographic investigation it can be seen that the same metabolites that are formed in the liver of the mother can also be found in the foetus. The *in vitro* investigation shows that nicotine can be meta bolized only in very small amount by the foetus and therefore the meta bolites in the foetus have been formed in the mother and transported via the placenta into the foetus.

Summary

The passage of ^{14}C-nicotine and its metabolites from the mother into the foetuses has been studied in mice. Nicotine and its metabolites are accumulated in the placenta and pass into the foetuses. The metabolites present in the foetuses obviously originate from the mother The passage of nicotine into the foetus has been shown to be the same during the last four days of pregnancy

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The Effect of Cytostatic Drugs on the Metabolic Response of Ascites Tumor Cells

By

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(Received August 14 1968)

Microfluorimetric studies (Chance & Thorell 1959) have revealed the specific responses of mitochondrial and extramitochondrial pyridine nucleotides (Kohen 1964) to metabolites added microelectrophoretically (Kohen, Kohen & Jenkins 1966) to the cytoplasm of various mammalian cells in culture (ascites cells, human liver cells, chinese hamster fibroblasts, primary explant cells) and even hexaploid yeast (Kohen Kohen & Thorell 1968 a b & c) In the past pharmacological studies with this method have been limited to drugs which exhibit the most obvious metabolic effects, such as Amytal ® (pentymal NFN) and rotenone (Ernster, Dallner & Azzone 1963) inhibitors of NADH-cytochrome b reductase, dicumarol (an uncoupler of oxidative phosphorylation cf Chance & Hess 1959) or iodoacetamide (an inhibitor of glyceraldehyde 3-dehydrogenase) The next step was to increase the reliability and sensitivity of the method, so that it could be used for observations with drugs or hormones which may have less drastic effects on the overall cellular metabolism but which might characteristically affect the kinetics of specific enzyme pathways in localized intracellular compartments.

In a preliminary phase, basic information was obtained about the relative contributions of the TCA cycle, Embden Meyerhof pathway pentose phosphate shunt etc. in the overall metabolic balance of various mammalian cells (Kohen Kohen & Jenkins 1966 Kohen Kohen & Thorell 1968 a & b) Also discrete metabolic differences were detected between nuclear and cytoplasmic pyridine nucleotides in terms of substrate and inhibitor sensitivity (Kohen Kohen & Thorell 1968 c) The development of a beam splitter system for cell manipulations synchronous with fluorescence recording (Kohen Kohen & Åkerman 1968) has contributed to the basic understanding of early kinetic processes after

microelectrophoretic addition of substrate On this basis it is now possible to start exploring pharmacological effects in a broader sense

As a starting point and in logical contribution to work previously done with irradiated cells (KOHEN KOHEN & THORELL 1968a), a radiomimetic (alkylating) drug was selected 2,3,5-triethyleneiminobenzoquinone, Trenimon (KUN, LANGER, ULRICH HOLZER & GRUNICKE 1964 HOLZER 1964).

Materials and Methods

The microfluorimetric-microelectrophoretic technique (KOHEN, KOHEN & JENKINS 1966) the ascites tumor tissue culture cells (EL2 cells, cf KOHEN 1964; KOHEN, SIEBERT & KOHEN 1964) and the X-ray produced EL2 giants (*EL2G* cells, cf. KOHEN, KOHEN & JENKINS 1966), the estimates relative to the amounts of substrates or metabolites introduced into the cytoplasm of single living cells (roughly 10^{-14} mole substrate/10 μμl cell volume) and the actual concentrations f reduced pyridine ucleotide reached in these cells have been described elsewhere (KOHEN, KOHEN & JENKINS 1966 KOHEN, KOHEN & THORELL 1968b &) I essence, the primary photocurrents corresponding to the blue fluorescence of reduced pyridine ucleotides (450 mμ) wer recorded from the photomultiplier and corrected for the "base-line value i.e. the mean value for substrate-free cells of the same experimental series.

The drugs used were an alkylating (radiomimetic) agent, Trenimon and malononitrile dimer 1 1,3-tricyanoaminopropene, which is stimulator f RNA synthesis (EGYHAZI & HYDEN 1961). Trenimon was used for short term experiments in the open chamber of the microfluorimeter (CHANCE & LEGALLAIS 1959 KOHEN & LEGALLAIS 1965) or for the production of giant cells (DEITCH & GODMAN 1967) by shock treatment of EL2 cultures (simultaneously with these experiments, Dr M Gonsalvez in this laboratory has produced giants in cultures of human amnion cells by treatment with N-desacetyl-methyl colchicine, actinomycin D etc.). For shock treatment with Trenimon ® (triazquon INN), the culture medium was removed and EL2 cells were incubated from 1 5 to 4.5 min. with 5 × 10^{-5} M solution of the drug. The solutio was then washed ff, and fresh medium replaced. This treatment leads to mass mortality but from survivors giants are formed, larger (60 × 60 μ) with the shortest exposure (1.5 min. Trenimon giants = *EL2T* cells), and not quite as large (40 × 40 μ) with the longest exposure (4.5 min. Trenimon giants = *EL2tr*).

Tricyanoaminopropene (TRIAP) was added nly in open chamber experiments. Since at the concentrations used (0.3 and 3 mM) the solution exhibited strong blue fluorescence, the drug was left in contact with the cells for 10 min. only and then the cells were washed with fresh medium. The drug did not become attached to cell membranes or structures in such way to alter the fluorescence readings by giving high background.

Pretreatment of cells and microelectrophoretic mixtures

Because of the relative difficulty in btaining high yields of Trenimon giants (EL2T nd EL2tr), the experimental conditions were set in such w y t enhance the possibility of significant fluorescence responses each time such giants could be obtained. Accordingly the comparative experiments between EL2T and EL2G cells were carried in the presence of Amytal which was previously observed to enhance the glycolytic response in ascites cells (KOHEN 1964). The glucose-6-phosphate (G6P) containing microelectrophoretic m

was supplemented with activators of phosphofructokinase (LOWRY 1965). Independent experiments have shown that the special combination f activators used (such as fructose-1 6-diphosphate (FDP) + adenosine d phosph te (ADP) or ADP + adenosine monophosphate (AMP)) did not matter significantly provided an denine nucleotide was in the mixture (KOHEN published observations). In greement with previous bservations (KOHEN, KOHEN & THORELL 1968 b & c), the substitution of a pyrimidine nucleotide, such as cyclic cytidine-5-monophosphate (cyc CMP) for adenine nucleotides did not alter the results. In these experiments for methodological reasons (KOHEN, KOHEN & JENKINS 1966), initial preference was given to EL2G controls because of thei giant size, but as the technique progressed it was gradually possible to include the standard size EL2 or other cells of the same size in these studies.

Kinetic measurements validation and specificity of fluorescence studies.

The beam splitter design which can reflect the red light used fo cell and microinstrument visualization t wards the ocular while transmitting towards the photocathode the blue light coming f om fluorescent intracellular structures, allows the continuous recording of the fluorescence in a selected intracellular compartment before, during and after microelectrophoretic ddition of substrate. Recent kinetic experiments, using this design (KOHEN, KOHEN THORELL & ÅKERMAN 1968) allow a reevaluation of previous results. They reveal that part of the rotenone- or Amytal-enhancement described previously (KOHEN 1964; KOHEN KOHEN & THORELL 1968 a & b &c see also preceding sectio on Pretreatment) may be due to a 10–15 sec. dealy in fluorescence recording after substrate addition (In experiments with n older design f the microfluorimeter CHANCE & LEGALLAIS 1959) the fluorescence response might have been already fading in the untreated EL2, while still close to peak level in Amytal- rotenone-treated cells

The recorded blue fluorescence has two components a unchanging" background fluorescence which is non-specific and a fluorescence changing characteristically according to the metabolic state of the cell. The specific association of metabolically changing blue fluorescence with reduced pyridine ucleotides, has been discussed elsewhere (CHANCE & THORELL 1959). In its present stage, the microfluorimeter does not allow a direct distinction between NADH and NADPH However separation of NADH and NADPH is possible by the use of substrates specifically linked with one or other coenzyme and inhibitors specifically blocking NADH or NADPH-linked pathways (KOHEN, KOHEN & JENKINS 1966 KOHEN, KOHEN & THORELL 1968).

Since the fluorescence f reduced pyridine ucleotides sharply depends on the nature and quality of their binding (ESTABROOK 1962) it seems necessary to validate the fluorescence measurements by direct extraction and assay of reduced pyridine nucleotide (BORST & COLP BOONSTRA 1962 MAITRA & CHANCE 1965) nd calibration of these results with the intracell lar conditions of fluorescence (KOHEN, SIEBERT & KOHEN 1964 MAITRA & CHANCE 1965). Comparative fluorescence meas rements between intracellular pyridine ucleotides and glass capillary standard filled with known solution of reduced pyridine nucleotides are made (KOHEN, LEGALLAIS & KOHEN 1966). From such observations, plus the assays made by other investigators in extracts of ascites cells, it is possible t evaluate the intracellular concentrations of NADH which re dealt with I microfluorimetry and to estimate their bound state on the basis of fluorescence enchancement (ESTABROOK 1962). As the bove calibrations cannot all be made on same cell, further safeguard is to use each cell as its own control, for kinetic measurements before during and after substrate addition, so that rather than absolute fluorescence magnitudes it is the relative changes that are significant.

Table 1

Primary photocurrents recorded from the extramitochondrial region before and after microelectrophoretic addition of substrate ± standard error of the mean (S.E.M = $\pm\sqrt{\Sigma d^2/n(n-1)}$).

Cell type and treatment	Primary photocurrents (10^{-17} Amp.)		No of cells
	Before substrate addition	After substrate addition	
EL2[1])	2.9 ± 0.4	7.4 ± 0.3	14
EL2[1]) (Amytal, 1 mM)	3.1 ± 0.6	5.5 ± 0.1	8
EL2[1]) (Rotenone, 5 × 10^{-7} M)	3.3 ± 0.6	5.3 ± 0.2	7
EL2G[1]) (Untreated)	3.0 ± 0.3	5.4 ± 0.3	18
EL2G[1]) (Amytal, 1 mM)	3.6 ± 0.4	5.1 ± 0.1	10
EL2G[2]) (Tricyanoaminopropene, 0.3 mM)	3.3 ± 0.8	6.1 ± 0.5	14
EL2G[2]) (Tricyanoaminopropene, 3 mM)	2.9 ± 0.5	4.7 ± 0.3	15
EL2G[2]) (Trenimon, 0.01 mM)	3.5 ± 0.5	5.0 ± 0.3	16
EL2n	3.8 ± 0.4	4.5 ± 0.1	9
EL2n[2]) (Rotenone, 5 × 10^{-7} M)	3.5 ± 0.5	4.8 ± 0.3	11
EL2T[2]) (Amytal, 1 mM)	3.6 ± 0.4	5.6 ± 0.4	8

[1]) Maximal values recorded in the beam splitter microfluorimeter used for continuous fluorescence measurements, synchronously with cell manipulations, KOHEN, KOHEN, THORELL & ÅKERMAN 1968.

[2]) Experiments in the original microfluorimeter of CHANCE & LEGALLAIS 1959 where fluorescence responses to added substrat are recorded with a time lag (see methods). For radiation and drug giants, in which the peak fluorescence response remains at steady state level for a rather long time, determinations are not significantly altered by the time lag.

Results

Short term experiments in the open chamber of the microfluorimeter

EL2G cells were incubated with TRIAP (table 1) and the fluorescence was then recorded from the nucleus and cytoplasm before and after microelectrophorectic addition of a G6P containing substrate mixture (G6P+ cyc CMP). The control EL2G cells responded to glycolytic substrate by a 64 % increase of blue fluorescence in the nucleus and a 58 % increase in the cytoplasm After pretreatment with 0.3 and 3 mM TRIAP the nuclear and cytoplasmic responses were respectively +64 % and +103 % with the former and +56 % and +69 % with the latter dose.

Trenimon (10^{-5} M) was added to cells treated with G6P+ADP+AMP Using this mixture, the fluorescence responses in the simultaneous EL2G controls were +77 % in the nucleus, +85 % in the cytoplasm 10–15 min

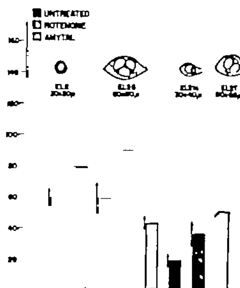


Fig. 1 Percent fluorescence responses i the extramitochondrial region of EL2 (first three columns from the left), the radiation giant EL2G (4th to 6th column), the Trenimon giants EL2ts (7th and 8th columns) and EL2T (last column), on addition of glycolytic substrate. The fluorescence level before substrate addition is arbitrarily equated to 100. The standard error of the mean calculated according to the formula S.E.M. $= \pm\sqrt{\Sigma d^2/n(\ -1)}$ is indicated on the left and top of each column.

after addition of Trenimon to the open chamber these same responses were respectively +39 / and +52 / (see also table 1)

Nuclear and cytoplasmic fluorescence responses in 4.5 min-Trenimon giants (EL2ts)

EL2ts giants showed an 18 / increase of fluorescence in the nucleus and a 19 / in the cytoplasm when treated with the same G6P mixture as above (G6P+AMP+ADP) These responses were 2–4 times lower than those observed in EL2G controls, and 7–8 times lower than the rapidly fading fluorescence response recorded in the untreated EL2 (table 1 fig. 1), but doubled on pretreatment with 5×10^{-7} M Rotenone.

Kinetic studies with Trenimon giants (EL2T) Irradiated cells (EL2G) and untreated EL2

In kinetic experiments with the beam splitter system, addition of glycolytic substrate (G6P+FDP+ADP) to EL2, EL2G and EL2T cells, by a microelectrophoretic current of 1–5 sec. duration, is followed by a

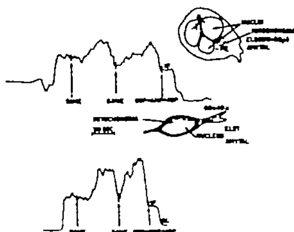


Fig. 2A. Microfluorimetric recording of the glycolytic responses in the extramitochondrial space of EL2G and EL2T in the presence of 1 mM Amytal. Th time scale proceeds from right to left. Increase of fluorescence is indicated as an upward deflection. BL = base line IF = initial fluorescence before addition of substrate. The amplitud f the peak fluore scence response is given by the maximum distance from the base line. The time scale and the magnitude of the primary photocurrent are indicated in 2B. For both EL2G and EL2T two consecutive fluorescence pulses are seen, f llowing two successive additions of a glycolytic mixture (G6P + AMP + ADP). Expts. BS67–7 and 8

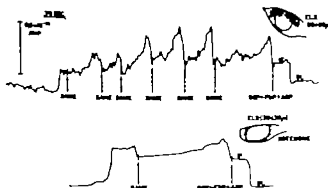


Fig. 2B. To provide a comparison with th kinetic changes bserved the drug & radiation giants, the microfluorimetric recordings f the glycolytic responses ntreated and rotenone-treated EL2 cells are shown. Rotenone-treatment is equivalent t Amytal treat ment in fig. 2A. The microelectrophoretic mixture in fig. 2A includes ADP and AMP as activators of phosphofructokinase, while in 2 B the activators are ADP and FDP. However independent experiments have evealed that the microelectrophoretic mixtures f figs. 2A and B are approximately comparable in the extramitochondrial fluorescence responses elicited in same cell type. (Corresponding cells are shown on the right upper corner of the tracings).

fluorescence pulse which starts almost instantaneously (figs. 2A and 2B), 1 to 5 sec after the initiation of microelectrophoresis or while it is still on. The increase of fluorescence occurs essentially in the extramitochondrial space (cytoplasmic and nuclear). The ascending phase of the pulse is rapidly terminated (1–9 sec.) and followed by a short plateau (5–10), before the fluorescence starts to decrease again returning to the original level. A second addition of substrate, may or may not result in a second fluorescence pulse of comparable magnitude. The overall pattern of the kinetic trace is quite comparable in EL2G and EL2T cells (fig. 2A), but in both it differs considerably from the rapid succession of fluorescence spikes exhibited by the untreated EL2 cells (fig. 2B). Fluorescence cycles with the longest periods are observed in the Rotenone-treated EL2 (fig. 2B). In untreated EL2 and EL2G cells up to 7–15 consecutive cycles may be obtained, but they exhibit longer periods in the irradiated giants. A maximum of three cycles could be recorded in Amytal-treated EL2, and of two cycles in Amytal treated EL2G and EL2T or rotenone-treated EL2.

The peak fluorescence response of the extramitochondrial space (cytoplasmic and nuclear summated) differed little in Amytal-pretreated EL2G and EL2T giants and was somewhat higher in similarly treated EL2 cells (+43 %, +56 % and +79 % respectively see also tables 1 and 2, fig. 1). Amytal-treated EL2G cells did not differ significantly from untreated or rotenone-treated EL2G controls. However the peak fluorescence response to G6P in the Amytal- (or rotenone-) treated EL2 cell was about half of that observed in untreated EL2 cells, but the latter faded more rapidly.

The response times were approximately comparable in all cell types investigated (table 2). However the duration of the ascending portion of the fluorescence curve was about twice as short in the untreated or Amytal treated EL2 cell (4–5 sec.) than in the corresponding EL2G and EL2T giants (8–9 sec.). The plateau phase at the peak fluorescence level lasted from 7–14 sec. in the giants, but only 4–5 sec. in the EL2 cells (table 2), although the amount of substrate added was adjusted to the cell size. From the standpoint of intracellular enzyme kinetics, the most significant value recorded in these experiments may be $t_{1/2 off}$ the time it takes for the fluorescence to rise and fall again to half maximal value. $t_{1/2 off}$ is respectively 9, 16, 22 and 42 sec. in untreated EL2 cells, Amytal-treated EL2, EL2G and EL2T (table 2). Among cells maintained exactly under the same conditions (Amytal-treated EL2, EL2G and EL2T), the shortest rise time, duration of plateau phase and $t_{1/2 off}$ are observed in the EL2 and the longest in the EL2T with the radiation giant EL2G occupying an intermediary position. The drug- and radiation-giants do not differ in rise time but the duration of steady state and $t_{1/2 off}$ are twice as long in the drug

Table 2

Kinetics f the fluorescence response after microelectrophoretic addition[1]) of a G6P containing glycolytic mixture.

Cell type nd treatment	Product of cell dimensions long its greatest nd smallest axes in the microscope (field (μ × μ)	Peak fluorescence increment (level prior to micro-electrophoresis = 100)	Response time (sec.)	Rise time (sec.)	Duration f plateau level (sec.)	$t_{1/2}$ sec	No. f cycles	No. of cells
EL2G (Amytal, 1 mM)	6000	+43 ± 4	1 7 ± 0 4	8 ± 1.6	7 ± 0.7	22 ± 3	1 2	8
EL2T (Amytal, 1 mM)	4000	+56 ± 10	1 6 ± 0.3	9 ± 1 6	14 ± 3 6	40 ± 6	1 2	8
EL2 (Amytal, 1 mM)	400	+79 ± 7	1 4 ± 0.3	5 ± 0.7	5 ± 0.7	16 ± 1	1–3	7
EL2 (Untreated)	400	+154 ± 18	1 6 ± 0 1	4 ± 0.5	4 ± 0.1	9 ± 1	1–6	14

[1]) The amount of substrate added to the cytoplasm f EL2 cells was 10–15 times maller th that given to EL2G EL2T which fits the volumetric relationship between EL2 cells and the radiatio or drug-giants (the cell thickness appeared relatively comparable in the EL2 and the giants).

giant. Furthermore in some EL2T cells the fluorescence did not fade completely after the rise due to addition of substrate and remained at a plateau higher than the initial level.

Discussion

The influence of alkylating agents on the extramitochondrial fluorescence response to glycolytic substrate

Whether EL2 or EL2G cells are used as a point of reference (and more so with the former than with the latter) there is an inhibition of the extramitochondrial fluorescence response in Trenimon treated cells or Trenimon-induced giants. In EL2ts giants which are the outcome of a more damaging exposure to the drug than EL2T giants the fluorescence responses are 7–8 times lower than in untreated EL2 cells and 2–4 times lower than in EL2G giants. In Amytal-treated EL2T cells, the peak fluorescence responses are significantly lower than in similarly treated EL2 cells, but practically the same as in EL2G. Despite their relatively high glycolysis, EL2G cells differ from untreated EL2, by their lesser sensitivity to Amytal or rotenone (fig. 1, table 1). Changes in their lipoprotein membrane structures (i.e. mitochondrial, cf. MANTEIFEL & MEISEL 1966) could account for profound alterations in their extramitochondrial (glycolytic) metabolism (MAITRA & CHANCE 1965, NEIFAKH *et al.* 1965, KOHEN & KOHEN 1966). The previously reported larger extramitochondrial responses in the EL2G as compared to the EL2 may be due in part to a more rapid fading of the fluorescence in the latter (see Methods). The estimation of total glycolytic activity in the time course of the fluorescence response, requires the integration of the total area delineated by the fluorescence pulse.

The glyceraldehyde phosphate dehydrogenase reaction was found to be inhibited in ascites cells treated with Trenimon. Microfluorimetric determinations 10–15 min. after addition of Trenimon to the incubation medium are consistent with previous experiments by GRUNICKE & HOLZER (1966) who reported a sharp fall in NAD content 15 min. after treatment with this same drug. The inhibition of the NAD synthetizing nuclear enzyme, NAD pyrophosphorylase (SIEBERT 1966) or the activation of the NAD destroying enzyme NAD glycohydrolase could equally account for the results (KUN *et al.* 1964, GRUNICKE & HOLZER 1966).

The effects observed with radiomimetics, can be compared to those seen after radiation. After radium (CRABTREE 1933) or X-irradiation (MANTEIFEL & MEISEL 1966) glycolysis can proceed unimpaired or may be even abnormally high possibly due to the failure of mitochondrial control

mechanisms (CRABTREE 1933 MAITRA & CHANCE 1965 KOHEN, KOHEN & THORELL 1968a & b & c), as mitochondrial (and nuclear?) oxidative phosphorylation systems are highly radiosensitive (MANTEIFEL & MEISEL 1966). However radiation studies by others (KLOUWEN 1966 SCAIFE 1966) with doses larger than needed for the production of giants (PUCK *et al* 1957) show inhibition of glycolysis due to NAD decrease as with the radiomimetics and with similar interpretations (GRUNICKE & HOLZER 1966). Can the decreased glycolysis of Tremmon-treated cells result from alterations in lipoprotein membranes, as presumably it occurs in irradiated cells? NAD glycohydrolase is found in the nuclear and microsomal fractions its activity may be inhibited by compartmentalization, as it may at least be partially localized at the inside of the membranes of the endoplasmic tubules (TICE & BARNETT 1952) Alkylating agents may cause a destruction of these membranes or alter their permeability (EMMELOT & BENEDETTI 1960), thus leading to activation of NAD glycohydrolase (GRUNICKE & HOLZER 1966)

The relevance of kinetic fluorescence studies to intracellular enzyme activity

Various cells may show similar or identical peak magnitudes of the fluorescence response and yet differ in one or the other of the kinetic parameters i.e. initial response and rise times, duration of plateau, $t_{1/2off}$ and number of cycles elicited by consecutive additions of substrate (table 2). An empirical formula can be used to determine intracellular enzyme activity (CHANCE 1952) if the added substrate x_0 and $t_{1/2off}$ are known enzyme activity $= \frac{x_0}{t_{1/2off}}$. There are considerable differences in terms of kinetic parameters between Amytal-treated EL2 cells on the one hand and similarly treated EL2G giants on the other There is in particular an inverse relationship between the magnitude of the fluorescence response and $t_{1/2off}$ (fig. 3). If from these findings any inference can be made to the intracellular activity of glycolytic enzymes (as substrate concentration x_0 was adjusted roughly to the cell size), they might indicate that the highest level of activity is found in the EL2, the lowest in the EL2T with the EL2G occupying an intermediate value. The interpretation of the results is somewhat complicated by the dependence of $t_{1/2off}$ on NAD reoxidation. So far there is no known direct effect of the alkylating agent on this reoxidation (HOLZER & KRÖGER 1958) In considering the extent of glycolytic inhibition, it should be noted that drug resistant cells do not reveal any changes in NAD level (HOLZER 1964) In the case of EL2T we are dealing with relatively resistant survivors.

The above experiments provide some insight about the overall activ

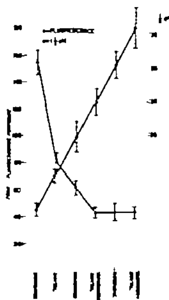


Fig. 3 The inverse relationship of the peak fluorescence response and t_{max} in ascites cells maintained and grown under a variety of conditions.

of glycolytic enzymes in these cells. The ultimate end should be to assess the influence of drug action on a single intracellular enzyme this hinges largely on reaching a pool of NADH in thermodynamic equilibrium with a single dehydrogenase of single known intracellular location (KOHEN, KOHEN, THORELL & ÅKERMAN 1968) While substrate selectivity would be required for the identification of a single dehydrogenase G6P which was used in these experiments can be catabolized through various NAD- or NADP linked pathways.

More accurate characterization of the coenzymes participating in the fluorescence process depends on the ability to record fluorescence spectra from localized cellular regions Also at long range, changes in conformation and orientation of the bound coenzyme (which might be due to bonds raising the energy level of the first electronic excited state, VELICK 1961) could be investigated by measuring the lifetime of the molecule in the excited state (PRINGSHEIM 1949)

The possible influence of alkylating agents on metabolic control mechanisms

In conclusion, with lethal doses of radiation or alkylating agents, there is a massive inhibition of glycolysis and a decrease of extramitochondrial (both nuclear and cytoplasmic) NAD possibly through damage to

lipoprotein membrane structures and loss of compartmentalization of degrading enzymes. With lesser doses giants are produced which exhibit more or less active glycolysis. These changes are consistent with previous reports of almost complete disappearance of NAD in the extramitochondrial space (cytoplasm + nucleus) after Trenimon-treatment (BORST *et al* 1963), while mitochondrial NAD remains unaffected Inhibition of nuclear glycolysis (following loss of nuclear NAD) can result in a block of RNA synthesis (MCEWEN, ALLFREY & MIRSKY 1964). On the other hand studies in phytohemagglutinin (PHA)-stimulated lymphocytes (POLGAR, FOSTER & COOPERBAND 1968) show that the increased DNA synthesis and cellular activation following PHA stimulation are dependent on glycolysis rather than respiration and furthermore that they are sensitive to inhibitors of glycolysis such as deoxyglucose. Therefore both the evidence from activators and inhibitors of cell division seem to indicate a possible causal relationship between glycolysis and the nucleoprotein metabolism of the cell.

Summary

A beam splitter supplemented Chance Legallais microfluorimeter uses the blue fluorescence and ubiquity of reduced pyridine nucleotides, for their kinetic assay in localized cellular fractions (e.g. mitochondria, cytoplasm or nucleus) during microelectrophoretic addition of substrates or metabolites. The system is particularly suitable for pharmacological studies in single living cells. Drug effects on intracellular enzyme activity can be evaluated by analyzing the fluorescence pulse resulting from addition of substrates, in terms of peak fluorescence response, halftime of fluorescence rise and decay number of cycles elicited by repetitive additions of substrate, etc. The glycolytic responses of the extramitochondrial pyridine nucleotides have been studied comparatively in untreated ascites cells in culture (EL2 cells in culture (EL2 cells) and giants produced by X-irradiation or treatment with an alkylating agent, 2,3,5-triethleneiminobenzoquinone 1 4 (Trenimon) So far the effects detected are in favour of a certain analogy between the effect of radiation and that of alkylating agents. With sublethal doses of radiation or alkylating agents, there is a massive inhibition of glycolysis and a decrease of extramitochondrial NAD With lesser doses giants are produced which exhibit more or less active glycolysis. An inverse relationship is found between the magnitude of the peak fluorescence response and its halftime. The highest peak fluorescence response to glycolytic substrate is found in the EL2, the lowest in the Trenimon-induced giant, with an intermediate value for the radiation-giant. The halftime of fluorescence rise and decay

is prolonged in radiation or Trenimon-giants with reference to the untreated EL2. These changes may be due to alterations in lipoprotein membrane structures (e.g. membranes of endoplasmic reticulum or mitochondria)

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The Fate of Atropine in the Dog

By

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A review of the literature reveals that there are important species differences both with regard to the metabolic disposition of atropine and the doses needed to produce its pharmacological effects. After the injection of ^{14}C-labelled atropine, mice excrete 80–90 % of the label within 48 hr Approximately 25 % of the radioactivity is in the form of unchanged atropine and over 50 % as conjugates with glucuronic acid (GOSSELIN *et al* 1955 GABOUREL & GOSSELIN 1958) In the guinea pig, on the other hand, most of the radioactivity in the urine is in the form of tropic acid (KALSER *et al* 1957). In man 50 % is in the form of unchanged atropine Little if any radioactivity was excreted as glucuronides and less than 2 % as tropic acid (GOSSELIN & GABOUREL 1958)

There are also large species differences with regard to the doses needed to produce a given pharmacological effect. Dogs are about ten times more sensitive with regard to the effects on the central nervous system than mice or rats (*see e g.* LONGO 1966)

In connection with studies on the effects of atropine on the central *nervous system of the dog* (ALBANUS *unpublished* ALBANUS *et al* 1967 & 1968 a), it became important to know the metabolic fate of the drug in this particular species.

Methods

Tritiated atropine[1] (generally labelled) with a specific activity of 172 mCi/mmol was used. The radiochemical purity was checked by descending paper chromatography using the upper phase of a solution of n-butanol, water acetic acid (5 5:1) and by high voltage electrophoresis in 0.1 M borate buffer at pH 10. The radioactive spots were localized with

[1]) Purchased from the Radiochemical Centre, Amersham, England.

a Packard radiochromatograph. A stock solution in dilute HCl containing 1–5 mCi/ml was stored in the deep freeze. The stability was frequently checked by paper chromatography

The study was undertaken in 15 beagles of both sexes weighing 10–15 kg. All injections were administered subcutaneously in the nape of the neck (50 µCi corresponding to 0.1 ml/kg). Different doses of atropine were obtained by adding unlabelled tropine sulphate.

When the distribution of radioactivity between the different parts of the brain was studied, the dogs were anesthetized with sodium pentobarbital and decapitated at the end of the experiment. After perfusion of the brain with 400 ml f saline through the carotid arteries, the different brain structures were dissected out, frozen in liquid nitrogen and ground to a fine powder

Th distribution of radioactivity between the plasma and the cerebrospinal fluid (CSF) was further studied in three dogs anaesthetized with halothane in mixture of O_2 and NO_2 (1 1) Cannulas were inserted into one lateral ventricle (using stereotaxic frame) and into the cisterna magna. In the epicerebral subarachnoid space small catheter (PE10) was introduced through a hole in the rostral part f the kull roof and the CSF was allowed to flow freely From the other compartments samples of 0.1 ml were collected at different time intervals. Blood samples were taken from the saphenous vein. The urinary bladder was catheterized.

Assay of radioactivity in tissues and body fluids

1 g of ground tissue (or 1 ml of heparinized plasma) was extracted with 2 ml acid ethanol (0.2% acetic acid in 96% ethanol) at room temperature for 30 min. and then centrifuged. The pellet was then re-suspended in 1 ml acid ethanol (0.15% acetic acid in 70% ethanol) extracted for 30 min. and centrifuged. The latter procedure was repeated once. The combined supernatants were concentrated to 1 ml and assayed by liquid scintillation in Nuclear Chicago model 720 Liquid Scintillation Counter T 0.5 ml of the extract were added 0.5 ml water and 14 ml of scintillation solution containing 960 ml dioxane, 5.12 g PPO 0.128 g POPOP and 102.4 g naphthalene. Quench corrections were made by the channels' ratio procedure. The efficiency and reproducibility of the extraction proced re was checked by adding known amounts f radioactive atropine to heparinized human plasma. The recovery was 89.5 % and th reproducibility ±1.8% (standard error f the mean of 14 experiments).

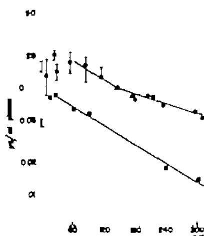
Urine and CSF samples were assayed by adding 10–400 µl of fluid to water (total volum 1 ml) followed by 14 ml f the scintillatio solution. Labelled metabolites in the urine were separated by paper chromatography and high voltage electrophoresis.

The binding f atropine to plasma proteins was determined by centrif ging plasma, to which a known amount f radioactive atropine had been dded by means of bags made of Visking" dialysis tubing (Unio Carbide Co., Chicago mean pore size 24 Å). Leakage of protein was checked by UV analysis accordi g to the method of ZAMENHOF (1959). The leakage was found t be less than 5%. The ultrafiltrate was assayed for radioactivity as described above.

Results

Absorption of atropine following subcutaneous injection

Atropine is rapidly absorbed into the blood following subcutaneous injection. Following a dose of 0.5 mg/kg (expressed as atropine sulphate) a maximum concentration in the plasma of about 0.2 µg/ml is reached



F g. 1 Absorption f tropine f llowing subcutaneous administration f ^{3}H-atropine. ● 0.5 mg/kg body weight (vertical bars indicat extreme values in five dogs), ■ 0.3 mg/kg and ▲ 0.1 mg/kg body weight. Th doses are expressed as tropine sulphate.

after about 25 min (fig. 1). Half this concentration is reached within ten min The elimination from the blood is slow and appears to be composed of two exponential functions as seen in fig. 1 Following a lower dose (0 3 mg/kg) elimination is more rapid due to the absence of the slow second phase.

Metabolic transformations of atropine

After the subcutaneous injection of 0 5 mg/kg about 30 / of the injected radioactivity is recovered in the urine after 2 hr., and 50 / is excreted within 6 hr (fig. 2). Paper chromatography of the urine in n butanol water acetic acid (5 5 1) and elution of the radioactive spots with water reveals that, after 2 hr 81–93 / of the excreted radioactivity

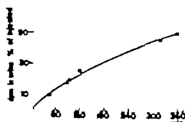


Fig. 2. Excretion f radioactivity in the urine following subcutaneous i jection of labelled atropine into t dogs.

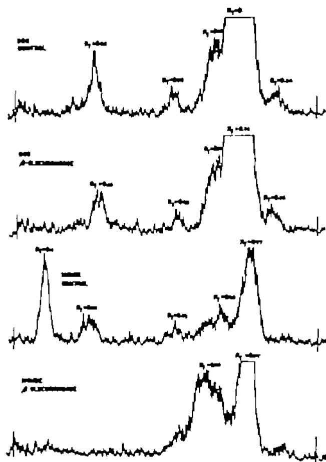


Fig. 3 Paper chromatography f radioactive metabolites in the urine from dog and mouse following injectio f tritium labelled tropine (? b orme)

is in the form of unchanged atropine (Rf = 0 70–0 77) (fig. 3). The composition of the urine with regard to radioactive metabolites is shown in table 1 It can be seen that the major metabolite (5–6 / of the radioactivity after 2 hr) has an Rf values of 0.25–0.32 (Metabolite I). In addition, varying amounts of metabolites with Rf values 0.49–0.55 (Metabolite II) 0 66–0 69 (Metabolite III) and 0 89 (Metabolite IV) are found

As seen in fig. 3 a radioactive glucuronide with the same Rf value as Metabolite I is excreted in mouse urine following the injection of ^{3}H atropine. However Metabolite I is not split by β-glucuronidase under these conditions when the glucuronide in mouse urine is split (fig. 3).

Metabolites II and IV are probably identical with tropine and tropic

Table 1

Per cent of radioactive atropine and metabolites in dog urine at different times after subcutaneous injection of 0.5 mg/kg (expressed as atropine sulphate) of ^{3}H-atropine (50 μCi/kg).

Dog No.	Collection time after injection min.	Metabolite I R_f = 0.25–0.32	II R_f = 0.49–0.55	III R_f = 0.66–0.69	IV R_f = 0.89	Atropine R_f = 0.70–0.77
1	0–30	–	5.1	4.8	2.3	81.0
2	0–120	6.6	–	–	–	93.4
3	0–120	5.2	0.9	–		92.0
4	0–360	9.8	2.8	5.9	–	67.0

Figures indicate per cent of the radioactivity spotted on the chromatogram

acid, respectively since they are chromatographically and electrophoretically indistinguishable from the products formed by alkaline hydrolysis of the tritiated atropine

Distribution

The distribution of radioactivity to different parts of the brain was studied at three different time intervals after 0.5 hr when according to a previous study (ALBANUS unpublished), the atropine effects on behaviour are not yet evident after 2 hr when the CNS symptoms are maximal and after 6 hr when the symptoms have disappeared The results are summarized in table 2 which shows the concentration of radioactivity at different time intervals. The data are expressed as ratio

$$\frac{\text{dpm in tissue per g}}{\text{dpm injected per g}} \times 100$$

The dose was 0.5 mg/kg. As seen in table 2, the concentration of radioactivity in the brain tissue at the different time intervals does not differ much. However the results indicate a relatively slow penetration into the brain. Thus, after 30 min. the concentration of radioactivity in the brain is only about $\frac{1}{2}$ of the plasma concentration, while the concentration in the heart is four times higher After 2 hr the concentrations in the brain and plasma are the same. It is also evident from the table that none of the brain structures studied contains a remarkably high or low concentration of radioactivity

Table 2

Concentration of radioactivity i different tissues following subcutaneous injection of tritium labelled atropi (0.5 mg/kg s c.) (Observations in seven dogs).

Tissue	Tissue concentration ratio¹)						
	0.5 hr		2 hr		6 hr		
Cerebral cortex	17	7	24	27	15	21	
C odate ucleus	22	23	32	27	22	25	20
Diencephalon	–	18	27	26	18	21	–
Mesencephal	28	17	30	33	20	19	
Metenceph lo							
pons	19	13	22	18	14	13	–
cerebellum	26	21	32	28	8	18	–
Myelencephal	24	27	24	19	8	17	–
Medulla spinalis	12	7	15	10	6	13	
Choroid plexus.		–	–		–	–	31
Liquo	–	–	–	–	–	–	26
Mand b lar gland		–	–	–	–	–	65
Retina		–	–	–	–	–	1,2
Ciliary body	–	–		–	–	–	164
Plasma	(30)		(25)	–	–	–	13
Blood		–	24	22	4	9	
Heart muscle	100	62	36	38	14	14	
Liver	241	232	321	268	56	85	
B le	–	460	2700	2500	14–30	15900	

The plasma values given in brackets are means from five dogs

$$^{1)}\ \frac{\text{dpm in tissue per g}}{\text{dpm injected per g}} \quad 10^2$$

It is interesting to note that, while after 6 hr the highest concentrations of radioactivity in the brain are about 2 times that in plasma, the concentrations in the salivary glands, retina and ciliary body are 5 to 10 times greater than the plasma concentration

Atropine enters the cerebrospinal fluid (CSF) and after 2 hr the concentration in the CSF is the same as in the plasma (fig. 4) After 10 hr it is about three times that of the plasma concentration The concentrations were the same in all the three compartments studied

The amount of radioactivity excreted in the bile is rather small. Thus 0 4 / of the injected radioactivity was found in the gall bladder after 30 min., 2.1 and 2.6 / after 2 hr and 12.0 and 16 7 / after 6 hr

Ultrafiltration of heparinized dog plasma to which labelled atropine was added *in vitro* showed that, at a plasma concentration of 0 03 µg/ml, less than 10 / is bound to plasma proteins.

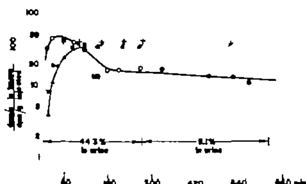


Fig. 4 Distribution of radioactiv ty between plasma and CSF f llowing subcutaneous injection of ^{3}H-atropine (0.5 mg/kg). O plasma. ▲ lat. ventricle. ■ cisterna magna + epicerebral subarachnoid space. The excretion f radioactivity in the urine is expressed in per cent f the mo nt injected.

Urinary excretion

The renal elimination was studied at different plasma concentrations. As seen in fig. 5 the amount of atropine excreted in the urine always exceeds the amount filtered by the glomeruli. Thus, the drug is secreted by the renal tubules. No Tm was reached at plasma concentrations up to 0.2 µg/ml Higher plasma concentrations could not be studied since the urine flow was strongly inhibited. In addition, the dogs became unmanageable because of the pharmacological effects of the drug.

At plasma concentrations below 0.1 µg/ml, there is a linear relationship between the plasma concentration and the rate of elimination. At higher plasma concentrations excretion is less efficient.

In three experiments, the effect of varying the pH of the urine on



Fig. 5. Urinary excretion f atropine (radioactivity) t different plasma

for the distribution and excretion of the drug. The fact that the urinary excretion is favoured by a low pH in the urine may have a practical application in cases of atropine intoxications. It must be remembered, however that we do not know what other effects acid base changes might have on the distribution of the drug in the body Thus, the shift of the blood towards the acid side following ammonium chloride administration is, in the CSF at the same time followed by a shift to the alkaline side (WINTERSTEIN & GÖKHAN 1953).

In the brain, the differences between the concentrations of atropine in different regions are relatively small The results are in agreement with those of VEIT & VOGT (1935) who used a bioassay technique.

The differences may be due to differences in the regional blood supply If our figures are compared with those of KETY (1966) for the regional blood supply in the cat, a direct correlation is indicated. There is no reliable evidence that atropine has any action which significantly alters the circulation through the brain (SOKOLOFF 1959)

The presence of atropine in the CSF after subcutaneous injection was already found in 1921 by STERN & GAUTIER by bio-assay The rapid appearance of a high concentration of radioactivity in the epicerebral subarachnoidal space in our experiments can not be explained by the bulk flow but indicates a passage of the drug from the meningeal vessels or from the extracellular fluid of the brain The remarkably constant concentration of atropine in the different compartments of the CSF shown in fig. 4 shows that the relation between in and outflux of the drug is the same between two and ten hr after the subcutaneous injection. No simple explanation can be offered for this finding. However the CSF concentration is about the same as that in the brain. It thus seems possible that the concentration in the CSF (higher than in plasma) is the result of an equilibration with the extracellular space of the brain. A decreased CSF production caused by atropine would also tend to maintain a constant drug concentration in the CSF A saturated active transport from plasma into the CSF via the choroid plexus would also explain the concentration plateau. However *in vitro* experiments with choroid tissue rather suggest a reverse direction of active transport for some quaternary and primary amines ((TOCHINO & SCHANKER 1965a & b)

The results of the present work show that the differences between mice and dogs regarding the sensitivity towards atropine can, to a great extent, be explained by the difference in the rate of metabolism. The fact that the pharmacological effects on the central nervous system are seen only after doses much higher than those which block peripheral cholinergic receptors is compatible with the uneven distribution of the drug.

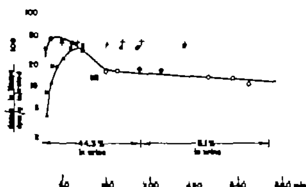


Fig 4. Distribution of radioactivity between plasma and CSF following subcutaneous injection f ³H-atropine (0.5 mg/kg) ○ plasma. ▲ lat. ventricle. ■ cisterna m gna. + epicerebral subarachnoid space The excretion f radioactivity in the uri is expressed in per cent of the amount injected.

Urinary excretion

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At plasma concentrations below 0 1 µg/ml, there is a linear relationship between the plasma concentration and the rate of elimination At higher plasma concentrations excretion is less efficient.

In three experiments, the effect of varying the pH of the urine on

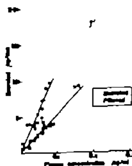


Fig. 5. Urinary excretion of atropine (radioactivity) at different plasma levels.

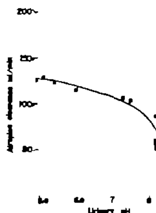


Fig. 6. Effect of urinary pH on tropine clearance.

atropine clearance was studied The results are summarized in fig. 6. A full record of a typical experiment is shown in table 3 The dogs were pretreated with 3 g NH_4Cl orally for three days before the experiment in order to bring the urine to a pH of about 5 0 The urinary pH could then be gradually increased by the infusion of $NaHCO_3$ The plasma concentrations in all cases were well below 0 1 µg/ml As seen in fig. 6 and table 3 atropine clearance is markedly influenced by the urinary pH. Net tubular transport is completely inhibited by pH levels in the urine of about 8

Table 3

Effect of urine pH tubular secretion f atropine.
The dogs were pretreated with 3 g NH Cl daily for three days. Alkalinization with sodium bicarbonate. GFR = reatinine clearance.

Time min.	Urine pH	Urine flow ml/min	GFR ml/min.	Atropine Plasma µg/ml	Atropine Filtered µg/min.	Atropine Excreted µg/min.	Atropine Secreted µg/min.
−53	300 ml H_2O orally						
0	Atropine 58 µg/kg (34 µc/kg) subcutaneously						
26–41.5	5.8	0.45	60.7	0.0.5	1.52	3 75	2.23
31 5–55.0	5.9	0.2	54.0	0.027	1 46	3.10	1 64
64	Prime 50 mmoles N HCO₃. Infuse 0.6 M $NaHCO_3$, 1 ml/min.						
55–70	7 8	1.50	65 0	0.028	1.82	2.58	0.76
70–85	8 1	5 13	58.3	0.026	1.51	1 49	−0.02
85–102	8.1	2.94	53.0	0.026	1.38	1.32	−0.06
102–117	8.1	2.87	65 1	0.024	1.56	1.47	−0 09

Discussion

According to the present study the major path of elimination of atropine in the dog appears to be urinary excretion of the unchanged drug. However plasma concentrations over 0.1 µg/ml produce a marked decrease in the net tubular secretion of the drug and this may explain the differences in the rate of elimination from the plasma after 0.3 and 0.5 mg/kg shown in fig. 1

Relatively small amounts were found in the bile as compared with results obtained in rats where 50 % of the radioactivity is excreted in the bile within 4 hr (KALSER *et al* 1965) These authors also showed that the biliary excretion is markedly increased following nephrectomy This may explain the high increase in the biliary excretion seen in the dog between 2 and 6 hr following 0.5 mg/kg when urinary excretion is diminished.

In mice, the identity of the metabolites excreted in the urine has been tentatively established (GOSSELIN *et al* 1955 GABOUREL & GOSSELIN 1958 WERNER 1961) The alkaloid is hydroxylated in the benzene ring in the para position (probably also to a certain extent in the meta position) and glucuronides are formed from both atropine and the hydroxylated atropine. In addition small amounts of tropine, tropic acid and tropine modified atropine are present. We have made no attempt to establish the identity of the four metabolites in dog urine. By comparing Rf values with the data in the literature it would appear that no qualitative differences exist between atropine metabolism in mice and dogs. One fact, however which provides some doubts regarding the validity of these superficial comparisons is that, while metabolite I has the same Rf values as a glucuronide of atropine found in mouse urine, the former is not split by β-glucuronidase under conditions prevailing when the latter is split.

Since not more than 10 % of the radioactivity in the urine is in the form of metabolites during the first two hr tissue concentrations of atropine have been calculated without any corrections being made for the metabolites. Furthermore in the mouse, where atropine is extensively metabolized, chromatographic seperation of radioactive metabolites in different tissues, has shown that the salivary glands, lung, brain and blood contain only a small percentage of metabolites as compared with the urine (ALBANUS *et al* 1968 b) It is thus reasobable to assume that the error introduced into the calculations of tissue concentrations is less than 10 %.

The finding that the renal elimmation of atropine is markedly influenced by the pH of the urine, that it is secreted into the stomach (ALBANUS *et al* 1968 b), and that it is absorbed from the stomach only when the contents are made alkaline (TONNESEN 1948), show that pH gradients are important

for the distribution and excretion of the drug. The fact that the urinary excretion is favoured by a low pH in the urine may have a practical application in cases of atropine intoxications. It must be remembered, however that we do not know what other effects acid base changes might have on the distribution of the drug in the body. Thus, the shift of the blood towards the acid side following ammonium chloride administration is, in the CSF at the same time followed by a shift to the alkaline side (WINTERSTEIN & GÖKHAN 1953).

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The results of the present work show that the differences between mice and dogs regarding the sensitivity towards atropine can, to a great extent, be explained by the difference in the rate of metabolism. The fact that the pharmacological effects on the central nervous system are seen only after doses much higher than those which block peripheral cholinergic receptors, is compatible with the uneven distribution of the drug.

Summary

The absorption metabolism, distribution and excretion of ^{3}H-labelled atropine have been studied in the dog. Following subcutaneous injection, 25 / of the injected radioactivity was recovered in the urine after 2 hr and 90 / of the excreted amount was in the form of unchanged atropine The results explain the relative sensitivity of the dog towards the effects of atropine.

Atropine was secreted by the renal tubules and the urinary elimination was markedly pH-dependent. The latter fact may have practical applica tion in the treatment of atropine intoxications.

In the brain substance, atropine appeared only slowly and only low concentrations were found. Particular attention has been paid to the distribution of atropine in different regions of the brain. However the relatively small discrepancies found might be related to differences in regional blood supply The atropine concentrations in the ventricular cisternal and epicerebral subarachnoid space were found to be the same

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Sleep Induction by Progesterone and Medroxyprogesterone in the Canary

By

Göran Wahlström

(Received J ly 25 1968)

The anaesthetic properties of progesterone and other steroids have been known for more than twenty years (SELYE 1941). A recent review is that by P'AN & LAUBACH (1964) In human subjects a state of som nolence, which was assumed to be related to anaesthesia, can be induced by progesterone (MERRYMAN *et al.* 1954) The possibility that progeste rone or its metabolites might have sleep inducing properties has also been investigated (HEUSER 1967 HEUSER *et al* 1967 GYERMEK 1967 GYERMEK *et al* 1967) although the distinction between sleep induc tion and anaesthesia is not always clear A method by which the self-selected rhythm of light and darkness can be recorded in the canary is, however well suited for studying the true sleep inducing properties of different substances since self-selected darkness is a measure of period during which these animals are asleep (WAHLSTRÖM 1964) In the present paper the results obtained with progesterone and medroxyprogesterone are presented. With both agents it was possible to induce a sleep-seeking behaviour

Method

The method has been described in detail elsewhere (WAHLSTRÖM 1964). The canaries (*Serinus canarius*) are kept singly in wooden cages. Each cage is lighted separately by common electrical bulb (75 W 220 V). This light is controlled by one of the two perches inside the cage in such manner that the light is extinguished when the bird uses this perch. The bird can thus choose between light and darkness. The other perch is used only for recording locomotor activity when the light is on in the cage. Male canaries btained from local dealers and trained for at least a month before the experiments started All the birds sed in these experiments were well adapted to the cages. W food (commercial canary seed mixtures) were available t all times.

The waking up has been used as the starting point for the calculation of the circadian period, which usually consists of one activity period and one rest period. Sometimes short rest periods (only those longer than 0.5 hours have been counted) are interspaced in the main activity. These rest periods are added to the main rest period ("gross activity and rest" WAHLSTRÖM 1964). The number of experiments in which one or more such divided periods were recorded were four out of 24 experiments with progesterone and seven out of 31 experiments with medroxyprogesterone. Such divided activity periods occurred in the activity period in which the active substance was given (circadian period number 0) in experiments with progesterone and in 3 experiments with medroxyprogesterone.

Medroxyprogesterone (INN) as the acetate and progesterone were dissolved in olive oil and administered orally by stomach tube. Two concentrations of progesterone were used (8 and 16 mg/ml). Doses of the lower concentration were given in two equal parts separated at intervals of 2 5 minutes. Medroxyprogesterone (Perlutex ®) was used in a concentration of 5 mg/ml. The progestogens were given either in the first half (AM) or in the last half (PM) of the activity period. In order to be ble to divide the experiments into two series with regard to the time of progestogen dministration few experiments were performed around the middle of the activity period.

In the circadian periods before and after the progestogen administration, olive oil was given in the same amount and in the same way as the active substance. This placebo treatment could not always be carried out but in birds accustomed to the treatment placebo administrations have no effect on the circadian hythm (WAHLSTRÖM 1964).

In all experiments pre-experimental period of five circadian periods was first recorded. Progesterone or medroxyprogesterone was given as a single dose during the activity in the circadia period number 0 Behavioural changes were estimated by direct observation at irregular intervals after the progestogen administration. For each experiment the induced changes in circadian rhythm were calculated as differences from the pre-experimental average. Data from corresponding circadian periods in similar experiments were pooled. Average changes were calculated for duration of activity rest and period-length.

Results

A. *Experiments with progesterone*

The AM-series, in which progesterone (300 mg/kg) was administered orally as a single dose early in the activity consisted of 15 experiments. These experiments were performed on 5 birds. One of these birds was a confirmed female The same birds were used in the PM-series (9 experiments) in which progesterone was administered late in the activity The time interval between two successive experiments in the same bird was 2 weeks or more, except in three experiments where the time interval was 7–10 days. Four experiments were excluded for technical reasons.

There was no apparent difference between the behavioural changes induced by progesterone in the AM and PM-series. The most marked behavioural change was slight difficulty in using the perches, "drunkenness" Such a change was noticed in 6 out of 16 experiments where there were enough observational data to make an evaluation possible. No case of anaesthesia was recorded. The "drunkenness" usually appeared within

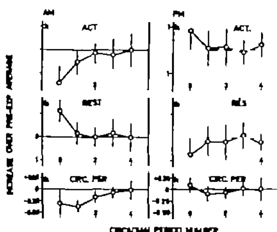


Fig. 1 The effects of a single oral dose of progesterone (300 mg/kg) on the circadian rhythm. The dose was given either during the first half (AM) or last half (PM) of the activity The number of experiments was 15 in the AM-series and 9 in the PM-series. Increases were calculated from the averages f the 5 circadian periods prior to the hormone administration in period number 0. Vertical bars indicate twice the S.E.M. The hormone was on an average given 2.60 hr (AM-series) and 8.68 hr (PM-series) after waking up in circadian period number 0. The mean pre-experimental (Pre-Exp.) average for all experiments in the AM-series was: activity (Act.) 12.08 hr rest 11.88 hr and circadian period (Circ. Per) 23.95. In the PM-series the corresponding values were 11.08 hr 12.49 hr and 23.57 hr

0.25 hours after the drug administration and lasted approximately one hour Some sedation was also recorded in most of the experiments with adequate observational data. In most of the experiments the sedation was apparent at least 1–2 hours after coordination had returned to normal. These last figures are, however very approximate as they are based on a subjective uncontrolled evaluation

The results of the AM and PM-series are given in fig. 1 When 300 mg/kg of progesterone was given early in the activity (AM-series) in circadian period number 0 the duration of the activity period was clearly reduced, due to an earlier roosting.

A corresponding, but a slightly smaller increase was seen in the duration of the rest period. The duration of circadian period number 0 was decreased The duration of circadian period number 1 was decreased an approximately equal amount although the changes in activity and were much less pronounced. A slight reduction was also seen in period number 2 where no changes in activity and rest were

In the PM-series (fig. 1) there was a slight increase in slight decrease in rest in circadian period number 0. These

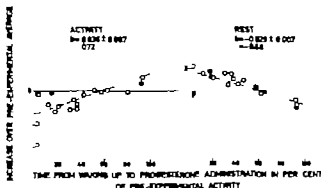


Fig. 2. The influence of the time between waking up at the start of the circadian period number 0 and progesterone administration, on changes induced in the duration of activity and rest in circadian period number 0. The time between waking up and progesterone administration is given as per cent of the average duration of the pre-experimental activity for each experiment. Squares denote experiments performed in a female. The dashed lines indicate the linear regressions. b = linear regression coefficient, r = correlation coefficient. The points with values smaller than 50 per cent belong to the AM-series.

the opposite of those seen in the AM-series. No definite changes could be seen in the duration of the circadian period. Fig. 1 thus shows that the response to progesterone clearly depends on the time in the circadian period at which the drug is administered This is further illustrated in fig. 2 and 3

Fig. 2 shows the relationship between the time from waking up to drug administration, expressed as per cent of the duration of the pre-experimental activity and the induced change in the duration of activity and rest in circadian period number 0 The data for the female experiments were not markedly different from the other experiments. A tendency towards greater variability was seen in the three female experiments performed immediately after waking up The data are, however too limited to state whether this is more than a coincidence In the experiment to the extreme right in fig. 2 the activity period had already lasted 1 6 hours longer than the pre-experimental average when the drug was given The position of this point on the time scale is thus debatable. There is, however a clear linear regression between the time from waking up to dose administration and changes in activity and rest. The changes in rest were the opposite to the ones in activity Both regression coefficients were significantly different from 0 ($P < 0\ 001$). The intercept between the regression line and the line of zero effect occurred when 58 8 (activity) or 57 5 (rest) per cent of the pre-experimental activity had elapsed at the time of drug administration.

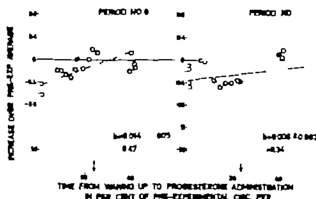


Fig. 3 The influence of the time between waking p starting circadian period umber 0 and progesterone administration, on the cha ges induced in the duratio of circadian period number 0 and 1 The time between waking up and progesterone dministration is given as per cent of the verage duration of the pre-experimental (Pre Exp.) circadian period for each experiment. Squares denot experiments performed in a female. The dashed lines indicate the linear regressions, b = linear regression coefficient, = corre lation coefficient. The points with values smaller than indicated by the arrow belong to the AM-series.

Fig. 3 shows the relationship between the time from waking up to drug administration (as per cent of the pre-experimental circadian period) and the changes induced in the duration of circadian periods number 0 and 1 A significant regression was found in circadian period number 0 but not in circadian period number 1 In circadian period number 1 the induced change was approximately the same in all AM-experiments. The changes in circadian period number 0 were not correlated to the changes in circadian period number 1 (r = 0 04 DF = 22)

The regression and correlation coefficients between the pre-experimental averages of the duration of activity rest and circadian period and their corresponding values influenced by progesterone in circadian periods number 0 and 1 are given in table 1 As expected (the induced changes were smaller than the differences between the experiments) there was a very high correlation in all sets of data All regression coefficients except two however were close to one. A regression close to one means that the value of the pre-experimental average did not influence the of the progesterone induced changes. The two exceptions in the F series consisted of activity and rest in circadian period number 0 data were, however few in the PM-series and in one experiment spontaneously increased activity If this value is excluded the coefficients with regard to activity and rest are 0.75 ± 0 06

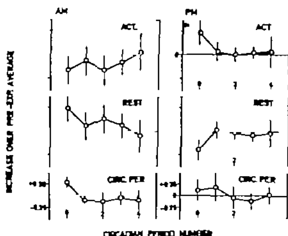


Fig. 4. The effects of single oral dose of medroxyprogesterone (75 mg/kg) on the circadian rhythm. The dose was given either during the first half (AM) or last half (PM) of the activity The number of experiments was 17 in the AM-series and 14 in the PM-series. Increases were calculated from the averages of the 5 circadian periods prior to the drug administration in period number 0. Vertical bars indicate twice the S.E.M. The hormone was on an average given 2.51 hr (AM-series) and 8.46 hr (PM-series) after waking up in circadian period number 0. The mean pre-experimental (Pre-Exp.) average for all experiments in the AM-series was: activity (Act.) 11 40 hr rest 12.60 hr and circadian period (Circ. Per) 24.00 hr In the PM-series the corresponding values were 11.35 hr 12.86 hr and 24 11 hr

the female experiments were clearly of the same magnitude and similar to the other experiments. A slight tendency towards a larger scatter in the experiments in which medroxyprogesterone was given close to the waking up time can be seen. There were, however few experiments in medroxyprogesterone was given approximately in the middle of the activity period. The linear regression coefficients for the activity and rest were calculated on the combined AM and PM-series and these coefficients *were significantly ($P < 0.001$) different from zero Changes in duration* of activity were the opposite to those observed in the duration of rest. The linear regression coefficients were of the same magnitude as the ones obtained in the progesterone experiments (fig. 2)

Fig. 6 shows the relation between the time from waking up to drug administration (as per cent of the pre-experimental circadian period) and the changes induced in the duration of circadian periods number 0 and 1 None of the changes were related in a linear manner to the time of medroxyprogesterone administration The changes in circadian period number 0 seemed to be at a minimum when the drug was administered shortly after the middle of the activity Whether a corresponding maxi-

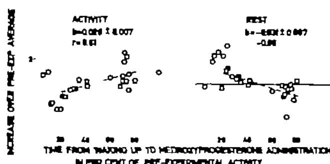


Fig. 5 The influence of the time between waking up starting circadian period number 0 and medroxyprogesterone administration, on the changes induced in the duration of activity and rest in circadian period number 0. The time between waking up and medroxy progesterone administration is given as per cent of the average duration of the pre-experimental (Pre-Exp.) activity for each experiment. Squares denote experiments per formed in females. The dashed line indicates the linear regressions. b = linear regressio coefficient, = correlation coefficient. The points with values smaller than 50 per cent belo g to the AM-series.

mum exists in the changes in circadian period number 1 is much more doubtful. There was no correlation between the changes in circadian period number 0 and those in circadian period number 1 ($r = -0.21$ DF = 29)

As can be seen from table 2 there was, as expected, a high correlation

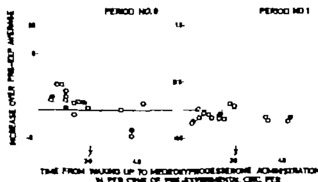


Fig. 6. The influence of the time between waking up i circadian period number 0 and medroxyprogesterone administration, on the changes induced in the duration of circadian periods number 0 and 1. The time between waking up and medroxyprogesterone administration is given as per cent of the average duration of the pre-experimental (Pre-Exp.) circadian period (Circ. Per.) for each experiment. Squares denote experiments performed females. The points with values smaller than indicated by the arrow belong to AM-series.

Table 2

Regression coefficients (b) and correlation coefficients (r) between pre-experimental average (x) and medroxyprogesterone influenced variables (y).

	y	AM-series (DF = 15)		PM-series (DF = 12)	
		b	r	b	r
Pre-experimental activity	Activity in circadian period no. 0	1.08 ± 0.22	0.79	0.82 ± 0.19	0.77
Pre-experimental rest	Rest in circadian period no. 0	1.26 ± 0.27	0.77	0.87 ± 0.12	0.90
Pre-experimental circadian period	Duration of circadian period no. 0	1.07 ± 0.09	0.95	0.84 ± 0.14	0.86
Pre-experimental circadian period	Duration of circadian period no. 1	0.97 ± 0.06	0.98	1.41 ± 0.17[1]	0.92

DF = degrees of freedom.

[1] The difference from 1.00 had a P value of < 0.05

between the pre-experimental average of the duration of activity rest and circadian period and the corresponding values in the circadian periods number 0 and 1 which were influenced by medroxyprogesterone. None of the regression coefficients were substantially different from 1 This means that the pre-experimental value of activity rest and circadian period did not influence the magnitude of the changes induced by medroxyprogesterone. With one exception, none of the regression coefficient in table 2 differed significantly from the corresponding values in the progesterone experiments shown in table 1 The exception was the regression between the pre-experimental circadian period and the duration of circadian period number 1 in the PM-series.

Discussion

Several steroids, among them medroxyprogesterone, progesterone and metabolites of progesterone, are known to induce anaesthesia (SELYE 1941 P'AN & LAUBACH 1964 MEYERSON 1967 GYERMEK *et al* 1967) Since many hypnotic drugs have anaesthetic properties in higher doses, for instance the barbiturates, there is a tendency to regard sleep inducing and anaesthesia inducing properties as being related, and only dependent on the dose. The common use of "sleeping time" instead of "time of absent righting reflexes" is one sign of this tendency The present method gives an opportunity of studying whether a drug can induce sleep clearly differentiated from anasthesia and at a time when sleep does not occur in normal animals, i. e. whether it can induce a sleep-seeking behaviour (WAHLSTRÖM 1964 & 1965). This is a situation clearly different from the therapeutic use of hypnotics where the drug is given to facilitate falling asleep at a time when the patient normally should go to sleep Several barbiturates as pentobarbital and barbital (WAHLSTRÖM 1965) secobarbital and nealbarbital (WAHLSTRÖM unpublished) have been studied by the present method With these barbiturates it has not been possible to demonstrate a clear sleep-inducing effect as defined above even in doses close to those inducing anaesthesia. Both progesterone and medroxyprogesterone could, however induce earlier roosting in the canary (fig. 1 and 4) when the steroids were given early during the activity The doses were comparable to those of the barbiturates with regard to "drunkenness" Sleep induction as measured with the present method is thus not related to induction of anaesthesia.

In the cat EEG patterns associated with sleep occur after systemically administered progesterone (HEUSER 1967) and also after intracerebral application in the pre-optic area in chronically implanted

(HEUSER *et al.* 1967) In the latter study the behaviour was also recorded. This is necessary in EEG experiments since there is no absolute electro-encephalographic criterion for sleep (JOUVET 1967) The cat is, however not well suited for studies on induction of sleep as the normal activity period in this animal is divided by short naps (STERMAN *et al* 1965). Nevertheless, it is evident that sleep can also be facilitated and probably even induced by progesterone in mammals.

In the PM experiments, when the drugs were given shortly before the expected roosting, the roosting was delayed (fig. 1 and 4) There is a possibility that this delay could be caused by the "drunkenness" seen in some experiments. This factor however could only play a part in the progesterone experiments where 4 out of 9 experiments were performed less than two hours before the expected roosting. In the medroxyprogesterone experiments the corresponding figures where only 2 out of 14 experiments. Nonetheless, roosting was equally delayed.

Another possible explanation for the difference between the AM and PM-series (with both agents) is a biphasic effect of the agents. Such a biphasic effect of progesterone has been demonstrated with regard to oestrous behaviour in the rabbit (SAWYER & EVERETT 1959), and in the guinea pig (ZUCKER 1966) Still more interesting is the fact that Sawyer and co-workers have also shown such a biphasic response in the threshold of EEG arousal and EEG afterreaction (KAWAKAMI & SAWYER 1959 SAWYER *et al* 1966) After progesterone and medroxyprogesterone both thresholds were first reduced and later increased The duration of the first phase was a few hours. It is possible that there is a similar biphasic effect of progesterone and medroxyprogesterone in the present experiments. The reasons for the biphasic effect are not known. Possibly metabolites with a depressant effect accumulate in the second phase

There are indications from experiments with L triiodothyronine and ^{131}I (WAHLSTRÖM 1968a) and from experiments with experimental dark periods during the first part of the self-selected activity (WAHLSTRÖM 1965) that roosting is regulated by a mechanism which is at least partly independent of the mechanism regulating the waking up time. In the present experiments the changes in waking up times (seen as changes in the duration of the circadian period) were evidently independent of the changes in the roosting (seen as changes in duration of activity and rest) (fig. 1 and 4) This would fit in with the two different mechanism but the possibility that active metabolites interfere complicates the analysis.

Whether or not the observed effects are correlated with the endocrine effects can only be decided after experiments with a wider spectrum of steroids.

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